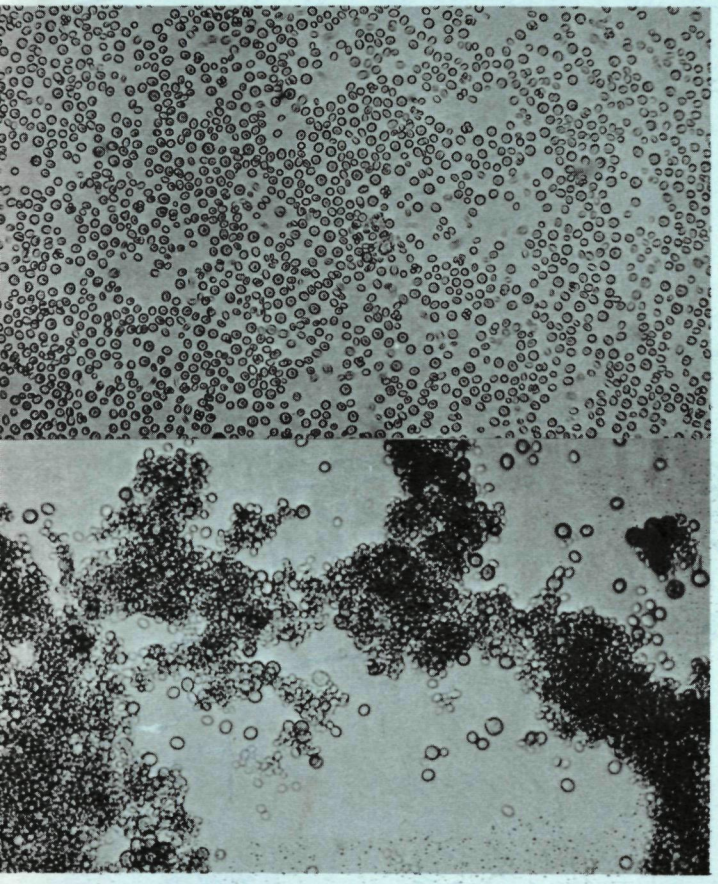
# OECOLOGISCHE EXPERIMENTEN MET TWEE GROENWIERSOORTEN



CHLAMYDOMONAS GLOBOSA

en

CHLOROCOCCUM ELLIPSOIDEUM

**H. W. Kroes**

# OECOLOGISCHE EXPERIMENTEN MET TWEE GROENWIERSOORTEN *CHLAMYDOMONAS GLOBOSA* EN *CHLOROCOCCUM ELLIPSOIDEUM*

Promotor: Prof. Dr. H.F. Linskens

# OECOLOGISCHE EXPERIMENTEN MET TWEE GROENWIERSOORTEN: *CHLAMYDOMONAS GLOBOSA* EN *CHLOROCOCCUM ELLIPSOIDEUM*

**PROEFSCHRIFT**

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
PROF. MR. F.J.F.M. DUYNSTEE,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 1 DECEMBER 1972
DES NAMIDDAGS TE 2 UUR PRECIES

DOOR

**HENDRIKUS WILHELMUS KROES**

GEBOREN TE NIJMEGEN

1 9 7 2
THOBEN OFFSET NIJMEGEN

Veel dank aan al degenen die op verschillende wijzen een bijdrage hebben geleverd aan dit onderzoek en dit proefschrift. In het bijzonder dank ik mej. J. Sterrenberg voor de nauwgezette uitvoering van vele experimenten en de heer J. Gerritsen en zijn medewerkers voor het verzorgen van de illustraties.

*Aan mijn ouders en mijn vrouw*

Overdrukken:
Limnology and Oceanography **16**, 869-879
Limnology and Oceanography **17**, 423-432
Archiv für Mikrobiologie **84**, 270-274

# INLEIDING

## 1. DOEL VAN HET ONDERZOEK

De oecologie houdt zich bezig met de relaties tussen levende organismen en hun omgeving: andere organismen en abiotische elementen. In tegenstelling tot de meeste andere biologische wetenschappen, zoals de anatomie, de fysiologie, en de systematiek, moet de oecologie haar gegevens dus per definitie voor een groot deel uit de vrije natuur betrekken. Werken in het veld heeft echter een methodisch nadeel: men heeft de waarden van de meeste voor de bestudeerde situatie belangrijke factoren niet in de hand, maar moet ze over laten aan de natuur zelf. Daardoor wordt het uitvoeren van experimenten ernstig bemoeilijkt. Men is dan ook bij veldwerk grotendeels aangewezen op het waarnemen en beschrijven van de situatie zoals die wordt aangetroffen, hetzij op een enkel moment, hetzij gedurende een langere periode. Uit grote reeksen waarnemingen kan men proberen conclusies te trekken over de werking van afzonderlijke factoren in het oecosysteem, al dan niet met behulp van modellen en wiskundige methoden.

Bij oecologische experimenten in het laboratorium kunnen de omstandigheden door de onderzoeker zelf worden gevarieerd. Hij is daardoor in staat het functioneren van de bestudeerde – eenvoudige – systemen sneller en directer te doorgronden. GAUSE (1934) legde veertig jaar geleden reeds de nadruk op de noodzaak van het bestuderen van 'elementaire processen in de strijd om het bestaan' onder gecontroleerde experimentele omstandigheden. Tot nu toe is er echter door oecologen te weinig geëxperimenteerd, misschien gedeeltelijk uit angst voor het uit het oog verliezen van de complexiteit van een oecosysteem. Talloze problemen uit het veld die voor een experimentele oplossing in aanmerking komen zijn nog tenauwernood onder handen genomen.

Bij de zoetwaterwieren met name is voor de meeste soorten de vraag: 'waarom komt de soort wel in het éne water voor en niet in het andere, en waarom komt de soort voor in combinatie met bepaalde andere soorten? ' bij lange na niet beantwoord. Zijn het voornamelijk anorganisch-chemische factoren (zouten, pH, $O_2$, $CO_2$) en fysische factoren (licht, temperatuur) die het voorkomen bepalen, of spelen ook directe relaties tussen wieren en andere organismen, of tussen wieren onderling, bijvoorbeeld via inhiberende stoffen, een rol? In sommige gevallen is het vrij gemakkelijk de belangrijkste verantwoordelijke factor te vinden, zoals bij halofiele en thermofiele soorten, maar voor de meeste andere zoetwaterwieren zijn er weinig precieze gegevens over de autoecologie. Onderzoekingen als die van RODHE (1948) en ÖSTERLIND (1949) over optimale zouten koolzuurconcentraties voor gekweekte wieren zijn schaars. De meeste studies hebben bovendien betrekking op een kleine groep veel gebruikte soorten: *Chlorella pyrenoidosa, Skeletonema costatum, Scenedesmus quadricauda,* enz. Door sommige onderzoekers is in het verleden veel waarde toegekend aan directe relaties tussen wiersoorten via inhiberende, 'antibiotische' stoffen (LEFÈVRE et al., 1951, 1952). Ook in recent werk van HARRIS (1971 a en b) wordt gesuggereerd dat antibiotische interacties tussen groenwiersoorten veel voorkomen.

In het onderzoek dat in dit proefschrift beschreven wordt is nagegaan in hoeverre er interacties tussen zoetwaterwiersoorten plaats vinden onder experimentele

omstandigheden en wat daarbij de relatieve belangrijkheid van verschillende factoren, abiotische en 'organische', is. Eencellige wieren lenen zich uitstekend voor experimenteel oecologisch werk omdat zc snel groeien, in een homogeen medium van precies gedefinieerde samenstelling gekweekt kunnen worden, en omdat het mogelijk is ze vrij te maken van alle andere organismen. Hoewel in dit onderzoek alle nadruk werd gelegd op de toepassing en ontwikkeling van experimentele methoden, werd toch geprobeerd een zekere binding te houden met de natuurlijke situatie, nl. door de wieren niet uit bestaande cultuurverzamelingen te bestellen, maar ze te isoleren uit een en hetzelfde biotoop, nl. het Ketelven (een der Hatertse Vennen in de omgeving van Nijmegen). Een tiental soorten, waaronder groenwieren, blauwwieren, en diatomeeën, werd in cultuur genomen. De opzet was om, na oriënterende proeven, hieruit een combinatie van twee soorten te kiezen voor een meer gedetailleerd onderzoek van interactieverschijnselen.

## 2. DE METHODIEK VAN INTERACTIEPROEVEN

De eenvoudige vraag: 'beinvloedt soort nr.1 de groei van soort nr.2? ' is dikwijls niet met dezelfde eenvoud te beantwoorden. Duidelijke gevallen zoals die van antibiotica producerende schimmels en de daarvoor gevoelige micro-organismen moeten in dit opzicht als uitzondering beschouwd worden. Bij gekweekte eencellige wieren is bijvoorbeeld aan naast elkaar groeiende kolonies op een agarplaat niet te zien of er onderlinge beinvloeding is; alleen nauwkeurige metingen van de groei van celpopulaties in een homogeen medium kunnen tot een gegronde conclusie leiden. Ook de vraag op welke wijze een groeiremming of -bevordering tot stand komt kan niet altijd met enkele simpele proeven beantwoord worden.

Door onderzoekers die zich in de afgelopen decennia hebben bezig gehouden met het bestuderen van interacties tussen wiersoorten, zijn in principe twee methoden gebruikt om vast te stellen of er beinvloeding optrad en waardoor deze werd veroorzaakt, nl. a) de mengcultuurmethode, en b) de filtraatmethode. In het eerste geval laat men twee soorten samen in één cultuur groeien en registreert men de groei van elk afzonderlijk: deze wordt vergeleken met de groei van controlecultures van de soorten apart. In het tweede geval wordt een filtraat van een cultuur van soort nr. 1 gebruikt als medium om soort nr. 2 in te laten groeien, al dan niet na aanvulling met voedingszouten. Tegen beide methoden in hun basisvorm zijn belangrijke bezwaren aan te voeren.

ad a) *De mengcultuurmethode,* die vooral bedoeld is om aan te tonen dat er interactie is, en om de vorm van deze interactie te kunnen beschrijven, stuit vooral op technische problemen. Voor de wieren die in dit onderzoek gebruikt werden kon geen bevredigende manier gevonden worden om de twee soorten in één cultuur afzonderlijk in hun groei te volgen. Drie methoden werden achtereenvolgens op hun bruikbaarheid onderzocht.

(i) Het tellen van de cellen onder een microscoop met behulp van een telkamer. Voor routinewerk bleek dit een tijdrovende en niet altijd betrouwbare methode te zijn. Niet alle soorten gaven bijvoorbeeld homogene suspensies: sommige, zoals *Chlorococcum*, hadden sterk de neiging om zich tegen de wand van de kolf vast te zetten of aggregaten te vormen. Bovendien waren de cellen van verschillende soorten niet in alle stadia goed van elkaar te onderscheiden.

(ii) Het tellen van de cellen met behulp

van een Coulter Counter Hoewel de nauwkeurigheid aanzienlijk groter was dan bij gewoon tellen, golden hier voor het overige dezelfde nadelen als onder (i) Bovendien konden alleen soorten met een duidelijk verschillende celgrootte van elkaar onderscheiden worden (de vorm speelt bij de discriminatie door de Coulter Counter niet mee)
(iii) Het meten van de O D (optische dichtheid) van de cultures bij verschillende golflengten Omdat verwacht mocht worden dat de pigmentsamenstelling van soort tot soort verschilde, zou de verhouding van de O D 's bij verschillende golflengten eventueel een maat kunnen opleveren voor de verhouding van de biomassa's der soorten in de mengcultuur Alleen bij combinaties van wieren uit verschillende groepen, bijv groenwieren en diatomeeen, werden echter enige successen geboekt Hoewel ook tussen groenwiersoorten verschil in pigmentsamenstelling werd gevonden, bleek dit te gering te zijn om de verhouding der soorten te bepalen

Het was dus onmogelijk in de mengcultures op een bevredigende wijze de groei van de twee soorten apart te volgen Naar twee kanten werd een oplossing voor het probleem gezocht Enerzijds werden mengcultuurproeven gedaan waarbij alleen de groei van de twee soorten samen werd geregistreerd en vergeleken met controle cultures Inleidende proeven op deze manier met acht soorten zijn beschreven in paragraaf 3 en soortgelijke proeven met *Chlamydomonas* en *Chlorococcum* zijn beschreven in het hierna volgende eerste artikel (Limnol Oceanogr 16, 869-879) Anderzijds werd een volkomen nieuwe methode ontwikkeld, de filtercultuur methode, waarbij twee soorten groeien in afzonderlijke cultuurvaten, maar praktisch in hetzelfde medium, doordat filtraat van de ene cultuur in de andere wordt gepompt en vice versa Ook deze methode en de hiermee verkregen resultaten zijn beschreven in het eerste artikel

Los van bovenstaande problemen kan men bij mengcultuurproeven op twee manieren kweken, nl met een open systeem de continu cultuur, of met een gesloten systeem de eenmalige cultuur (Eng batch culture) Zie hiervoor ook GAUSE (1934, p 6) In de continu cultuur laat men de soorten groeien onder constante toevoer van voedingsstoffen, in de eenmalige cultuur geeft men eenmaal bij het begin van de kweek, een hoeveelheid voedingsstoffen die later niet meer aangevuld wordt (afgezien van $CO_2$) Bij beide typen cultuur bereikt de populatiedichtheid op een gegeven moment een maximum bij de continu cultuur zodra het per tijdseenheid weggenomen percentage van de celpopulatie (bijv in een chemostaat) even groot wordt als het per tijdseenheid bijgegroeide percentage, bij de eenmalige cultuur zodra de voedingsstoffen op zijn of zich teveel schadelijke stofwisselingsprodukten verzameld hebben

In een continu cultuur met twee soorten die op dezelfde voedingsstoffen zijn aangewezen, zoals wieren die dezelfde zouten gebruiken, zal de ene soort de andere op den duur meestal volkomen verdringen ten gevolge van de regel dat onder gelijke omstandigheden een soort altijd wel iets harder groeit dan de andere ('het axioma der ongelijkheid' zie bijv HARDIN, 1960) Wanneer de omstandigheden (temperatuur, licht enz ) gewijzigd worden, kan de balans gemakkelijk naar de kant van de andere soort doorslaan Bekende voorbeelden van een dergelijke verdringing in experimentele oecosystemen zijn te vinden in het werk van GAUSE (1934) over *Paramaecium*-soorten, dat van PARK (1948 en 1954) over *Tribolium*-soorten, en dat van vele onderzoekers over *Drosophila*-soorten (zie bijv review van MILLER, 1967)

In een eenmalige cultuur kunnen beide

soorten tot een zekere maximum dichtheid groeien. Van verdringing is in deze opstelling geen sprake: alleen de groeisnelheid en/of de grootte van het bereikte maximum van elke soort kunnen door de andere beinvloed worden. In tegenstelling tot de situatie bij de continu cultuur kunnen in de eenmalige cultuur tijdens de groei sterke veranderingen optreden in de pH, de concentratie van voedingsstoffen, de aanwezigheid van stofwisselingsprodukten, enz.

De keuze van de methode – continu cultuur of eenmalige cultuur – zal voor elk onderzoek verschillend uitvallen, al naar de aard van de onderzochte organismen, de vraagstelling, en de beschikbare apparatuur. Ik heb in dit onderzoek steeds gebruik gemaakt van de eenmalige cultuur, en wel op grond van de volgende overwegingen.

(i) Bepaalde soorten, waaronder *Chlorococcum*, zetten zich vast op de wand van het glaswerk. Hoewel dit door roeren voor een groot deel kon worden voorkomen, bleef er steeds een zeker percentage vastgehecht. In een chemostaat-opstelling, waarbij per tijdseenheid een constant gedeelte van de cultuursuspensie wordt verwijderd, hebben wieren die zich vasthechten alleen al daardoor een voordeel op soorten die goed in suspensie gaan. Onderlinge beinvloeding via concurrentie om voedingszouten, door pH-effecten, of door afgegeven stoffen is in zo'n geval moeilijk te constateren.

(ii) Het feit dat de chemische samenstelling van het medium zich gedurende de groei in een eenmalige cultuur steeds wijzigt, maakt de interpretatie van de resultaten weliswaar gecompliceerder dan bij de continu cultuur, maar deze resultaten hebben een wat meer algemene betekenis doordat de soorten onder verschillende omstandigheden met elkaar hebben moeten concurreren.

(iii) De in dit onderzoek ontwikkelde filtercultuur methode is in de praktijk niet te combineren met een continu-opstelling.

ad b) *De filtraatmethode*, die in het verleden met name veel is toegepast om de aanwezigheid en werking van hypothetische remstoffen aan te tonen, moet theoretisch als zeer onbevredigend worden beschouwd. Een filtraat van een wiercultuur is ongedefinieerd wat betreft aanwezige voedingszouten. Zelfs als men een uitgebreide analyse van de anorganische bestanddelen zou uitvoeren (hetgeen door onderzoekers die de methode gebruikt hebben zelden of nooit gedaan is) zou het waarschijnlijk niet mogelijk zijn een controle medium van precies dezelfde anorganische samenstelling te maken (denk aan sporenelementen, pH, buffercapaciteit, enz.). Daardoor kan het effect van eventueel aanwezige groeibeinvloedende organische stoffen (door een wiersoort aan het medium afgegeven) onmogelijk gescheiden worden van het effect van de anorganische componenten. Op grond van deze overwegingen werd besloten geen totale filtraten te gebruiken voor interactietests. Wel werden organische stoffen uit de filtraten geisoleerd en in gezuiverde vorm, met name vrij van anorganische zouten, toegevoegd aan een vers medium en zo getest op groeibeinvloedende eigenschappen. Op deze manier kon tevens een indruk verkregen worden van de verscheidenheid aan stofgroepen die door de wieren worden afgegeven aan het medium en van de effecten die elk van deze groepen heeft op de groei van een andere soort. Het betreffende gedeelte van het onderzoek is beschreven in het tweede artikel (Limnol. Oceanogr. 17, 423-432).

## 3. SELECTIE VAN EEN SOORTENPAAR

Een aantal oriënterende proeven werd gedaan met als voornaamste bedoeling

een geschikt soortenpaar voor interactie-onderzoek te vinden. Deze werden gedaan vóórdat de wieren axenisch gemaakt waren: in de cultures konden zich dus nog bacteriën en eventueel schimmels bevinden. Dit leverde bij het kweken echter geen problemen op.

Erlenmeyers van 500 ml met elk 200 ml voedingsoplossing (samenstelling zie eerste artikel) werden geautoclaveerd en vervolgens geënt met twee te testen soorten. Per proef werden zes Erlenmeyers geënt: twee met soort nr. 1, twee met soort nr. 2, en twee met zowel soort nr. 1 als soort nr. 2. De cultures werden in een temperatuurkamer geplaatst en de groei der wieren werd gedurende anderhalf à twee weken gevolgd; voor details betreffende de kweekomstandigheden zie het eerste artikel. Voor het meten van de celdichtheid werden monsters van enkele ml genomen waarvan de O.D. bij 430 nm werd bepaald. Van de mengcultures werd dus, evenals van de aparte cultures (controles) de totale O.D. gemeten, d.w.z. er werd niet gedifferentieerd naar soort. De verkregen informatie was daardoor noodzakelijkerwijze aan beperkingen onderhevig, maar het voordeel van deze methode was een snelle en nauwkeurige bepaling van de groeicurves. De soorten voor deze proeven waren geselecteerd op eencelligheid; draadvormige typen zoals *Spirogyra* en *Ulothrix*, die oorspronkelijk ook geisoleerd waren, konden niet voldoende homogeen in het medium verdeeld worden. De volgende soorten werden getest: *Nitzschia* sp., *Gomphonema* sp., *Ankistrodesmus*, sp. 1, *Ankistrodesmus* sp. 2, *Scenedesmus* sp., *Kirchneriella* sp., *Chlorococcum ellipsoideum*, en *Chlamydomonas globosa*.

In totaal werden 13 combinaties van twee soorten getest. Elke combinatie leverde een stel van 3 x 2 volledige groeicurves op. Om overzichtelijk en gemakkelijk getallenmateriaal te verkrijgen werd als volgt te werk gegaan. Van elke curve werd de maximumwaarde genomen, dus de O.D. van het horizontale gedeelte van de meestal ongeveer S-vormige curve. Toevallige variaties werden zoveel mogelijk buiten beschouwing gelaten. De maximumwaarden van de duplo's werden gemiddeld, zodat voor elke proef drie getallen resulteerden: (i) de gemiddelde maximum O.D. van soort nr. 1 (A), (ii) de gemiddelde maximum O.D. van soort nr. 2 (B), en (iii) de gemiddelde maximum O.D. van de mengcultuur (M). Vervolgens werden A en B gemiddeld: ½(A + B). In tabel 1 zijn de gevonden waarden weergegeven.

Tabel 1

| soortencombinatie | ½(A+B) | M | verschil |
|---|---|---|---|
| Nit – Gom | 0,195 | 0,115 | + |
| An1 – An2 | 0,845 | 0,765 | + |
| An1 – Chc | 0,685 | 0,670 | + |
| An2 – Chc | 0,705 | 0,635 | + |
| | | | |
| Nit – An1 | 0,565 | 0,685 | – |
| Nit - Cha | 0,415 | 0,625 | - |
| Nit - Sce | 0,460 | 0,550 | – |
| Nit – Kir | 0,615 | 0,755 | - |
| Gom – An1 | 0,520 | 0,510 | + |
| Gom – An2 | 0,835 | 1,150 | – |
| Gom – Cha | 0,340 | 0,380 | - |
| Gom – Sce | 0,410 | 0,450 | – |
| Gom – Chc | 0,505 | 0,830 | – |

Bij het interpreteren van de resultaten werd uitgegaan van de volgende 'nulhypothese': in een mengcultuur van twee soorten die over evenveel ruimte en voedingsstoffen beschikt als elk der aparte cultures van deze soorten, zullen soort nr. 1 en soort nr. 2 elk slechts de helft van hun potentiële maximum dichtheid kunnen realiseren vergeleken met de controle cultures. Dit betekent dat M gelijk zal zijn aan ½(A + B). Een globale inspectie van

de tabel maakt duidelijk dat de nulhypothese in de meeste gevallen verworpen zal moeten worden Er werd geen statistische toets uitgevoerd, maar de spreiding in de duplo's was over het algemeen zodanig dat de gevonden verschillen niet toevallig tot stand konden zijn gekomen Bij enkele combinaties is de afwijking van de nulhypothese aanzienlijk (bijv Gom – An2 en Gom - Chc) Fen nadere beschouwing van de cijfers brengt een opmerkelijk feit aan het licht Bij alle combinaties van twee groenwieren of twee diatomeeen (de eerste vier in de tabel) is het gemiddelde maximum van de aparte cultures hoger dan het maximum van de mengcultuur Daarentegen is bij acht van de negen combinaties van een groenwier en een diatomee het gemiddelde maximum van de aparte cultures lager dan het maximum van de mengcultuur Deze summiere gegevens zijn natuurlijk niet voldoende voor een definitieve conclusie, maar voorzichtig kan gesteld worden dat diatomeeen en groenwieren niet door dezelfde factoren in hun maximum dichtheid beperkt worden en daardoor samen efficienter een bepaalde hoeveelheid medium kunnen benutten dan ze elk apart doen Combinaties van diatomee met diatomee of groenwier met groenwier zijn kennelijk minder efficient dan de aparte cultures, hetgeen erop zou kunnen wijzen dat beide soorten van de combinatie in elk geval door dezelfde factor in hun groei gelimiteerd worden, maar bovendien het milieu ongunstig voor elkaar beinvloeden, los van rechtstreekse concurrentie om voedingszouten

Uit de bovenbeschreven proeven werd geconcludeerd dat een groenwier-groenwier of een diatomee-diatomee combinatie een gunstig object zou zijn om verschijnselen van groeiremming nader te bestuderen Ondertussen waren de acht soorten axenisch gemaakt en was de filtercultuur methode (zie paragraaf 2) zover ontwikkeld dat verdere combinaties hiermee getest werden Een daarvan was het soortenpaar *Chlamydomonas globosa* - *Chlorococcum ellipsoideum* De resultaten met deze twee wieren waren van dien aard, zowel wat betreft onderling remmend effect als wat betreft het gemak waarmee ze onder de gestelde omstandigheden gekweekt konden worden, dat besloten werd het verdere onderzoek aan deze soorten te wijden De resultaten zijn beschreven in de drie artikelen die hierna volgen

## LITERATUUR


Gause, G F 1934, The struggle for existence Hafner Publ Co , New York Reprint 1964

Hardin, G 1960 The competitive exclusion principle Science **131**, 1292 1297

Harris, D O , 1971 a, A model system for the study of algae growth inhibitors Arch Protistenk **113**, 230 234

Harris, D O , 1971 b, Growth inhibitors produced by the green algae (Volvocaceae) Arch Mikrobiol **76**, 47-50

Lefevre, M , Jakob, H , en Nisbet, M , 1951, Compatibilites et antagonismes entre algues d'eau douce dans les collections d'eau naturelles Verh Int Ver Limnol **11**, 224-229

Lefevre, M , Jakob, H , en Nisbet, M , 1952, Auto- en heteroantagonisme chez les algues d'eau douce in vitro et dans les collections d'eau naturelles Ann Sta Cent Hydrobiol Appl **4**, 5-198

Miller, R S , 1967, Pattern and process in competition In J B Cragg (ed ) Advances in ecological research Vol 4, p 1-74 Acad Press, New York

Osterlind, S , 1949, Growth conditions of the alga *Scenedesmus quadricauda* with special reference to the inorganic carbon sources Symb Bot Upsal **10**(3), 1-141

Park, T , 1948, Experimental studies of inter-

species competition I Competition between populations of the flour beetles, *Tribolium confusum* Duval and *Tribolium castaneum* Herbst,Ecol Monogr **18**, 265-307

Park, T , 1954, Experimental studies of interspecies competition II Temperature, humidity, and competition in two species of *Tribolium* Physiol Zool **27**, 177-238

Rodhe, W , 1948, Environmental requirements of fresh-water plankton algae Symbol Bot Upsal **10**(1), 1-149

Reprinted from LIMNOLOGY AND OCEANOGRAPHY
Vol 16, No 6, November 1971
pp 869–879
*Made in the United States of America*

# GROWTH INTERACTIONS BETWEEN *CHLAMYDOMONAS GLOBOSA* SNOW AND *CHLOROCOCCUM ELLIPSOIDEUM* DEASON AND BOLD UNDER DIFFERENT EXPERIMENTAL CONDITIONS, WITH SPECIAL ATTENTION TO THE ROLE OF *p*H

*H. W. Kroes*
Department of Botany, University of Nijmegen,
Driehuizerweg 200, Nijmegen, Netherlands

ABSTRACT

The influence of *Chlamydomonas globosa* and *Chlorococcum ellipsoideum* on each other's growth was studied with two different experimental techniques. The first is a newly developed filter culture method in which two separate cultures of different species are connected via a filtering system through which medium is exchanged while the cells themselves are kept separate Under these circumstances *Chlorococcum* inhibits the growth of *Chlamydomonas* but not vice versa

In the second technique, traditional mixed cultures, *Chlorococcum* inhibits *Chlamydomonas* only in unbuffered medium In medium buffered at *p*H 8 1 there is a slight stimulation of one or both species The role of *p*H in the relation between the species seems therefore to be very important

## INTRODUCTION

There are two possible approaches to an understanding of the functioning of ecosystems. The first—trying to describe the whole of a particular system and to discover the laws governing it—necessarily produces data of a very high degree of complexity The second approach, used here, that of trying to isolate a tiny piece of the network and unravel it, offers more exact methods and clearer results, however, it is more difficult to integrate such data into an explanation of the whole.

Many workers have experimented with simple ecological systems of two or a few species of microorganisms since Gause (1934), who first tried to fit experimental data from mixed cultures of yeasts and protozoa to theoretical models. Notwithstanding the simplified treatment of results, his work is fundamental. Some of the many studies of the effect of different species of algae on each other's growth are discussed here, but there are many others (e.g., McVeigh and Brown 1954, Jakob 1954, Berglund 1969). All of these, except Talling (1957), found some form of inhibition or promotion, often dependent on the culture technique. The effect of algae and bacteria on each other has also been described frequently (Emers 1956, Kain and Fogg, 1958, Vladimirova 1961, Vladimirova and Basaitova 1961, Jørgensen and Steemann Nielsen 1961, Maksimova and Fedenko 1965, Vela and Guerra 1966, Maksimova and Pimenova 1969). In several instances promotion of algal growth in the presence of bacteria was found, the bactericidal effect of algal cultures has been studied especially by the Russian workers A review of experiments with other organisms (bacteria, protozoa, etc ) under similar conditions is given by Hobson (1969). The many practical difficulties in such experiments have necessarily resulted in inadequate descriptions of these phenomena. However, I believe that not enough attention has been paid, in the design of experiments and the interpretation of the results, to the following questions

In general, there has been insufficient realization that many factors may be responsible for interactions between species in liquid cultures. For example, the competition for inorganic salts has scarcely been investigated Proctor (1957) found that *Chlamydomonas reinhardi* used mainly $NH_4^+$ ions, whereas *Haematococcus pluvialis* showed a greater preference for $NO_3^-$

ions as a source of nitrogen Hulburt (1970) calculated the effect of competition for nutrients by phytoplankton under natural conditions

Although light is vital to the growth of algae there has not been a single investigation in which the problem of "competition for light" in mixed cultures was touched

The role of gas supply in mixed cultures of *Chlorella* and *Scenedesmus* was investigated by Golueke (1960), although in his experiments bacteria were also present, there appeared to be an influence of $CO_2$ concentration on the species composition of the culture Nakamura (1963) showed the importance of $CO_2$ production by bacteria to algal growth in mixed cultures, and, conversely, of $O_2$ production of the algae for bacterial growth Lange (1967) also reported a favorable influence of bacteria on algal growth via their production of $CO_2$

It is surprising that so little work has been done on the role of *p*H in mixed cultures Of the investigators mentioned, only Proctor (1957) reported the effect of *p*H In cultures of *C reinhardi* the *p*H rises to about 10 from an initial value of 7, and this rise seems to be inhibitory to *H pluvialis* The lack of data about *p*H effects probably stems partly from the difficulty of finding suitable buffers. In no study on the interactions of algae, or algae and bacteria, has a buffered medium been used Inorganic buffers—phosphate and carbonate—have been used rather widely for algal media, but they have the disadvantage that the buffering salts are at the same time nutrients for the algae There are few data on the use of organic buffers, Provasoli and Pintner (1960) discussed the characteristics of tris (hydroxymethyl) amino methane (Tris) triethanolamine (TEA), histidine, and glycylglycine as buffering substances for some freshwater species of algae, Reichart (1967) used Tris in cultures of *Spirogyra* but in a very low concentration (9 6 mg/liter)

The role of organic substances liberated by living or dead cells has been the central theme in many investigations, but only Jakob (1961) described a defined substance, a dihydroxyanthraquinone, considered responsible for the inhibition of algae growing in the filtrate of *Nostoc muscorum* Many investigators have, in my opinion, too easily accentuated the importance of excreted organic products above the importance of the "nonorganic" factors (*see* Hartman 1960, Lefèvre 1964)

Other objections that can be made to the experimental design of several investigations include, for example, the fact that the species selected for interaction experiments were often not isolated from the same locality In some early investigations, the cultures were not axenic (e g, Lefevre et al 1951, 1952) Even more recently, Levina (1964) and Pratt (1966) worked with nonaxenic cultures, their assertion that bacteria played no important role in their experiments seems incautious, especially in view of the findings of Ruschmann (1956) In several studies the culture filtrates used were prepared using ordinary filter paper (Lefèvre et al 1951, 1952, Jørgensen 1956, Jakob 1957) Filtrates obtained in this way might still contain particles such as cell debris and, in the case of nonaxenic cultures, bacteria

The aim of this study was to investigate interaction phenomena in algae, avoiding some of the above shortcomings In the course of preliminary investigations it became clear that conventional methods gave inconclusive answers, and a new method was therefore developed called the "filter culture method" The combination *Chlorococcum ellipsoideum-Chlamydomonas globosa* was chosen to be studied in detail

In this paper the results obtained with filter culture and mixed culture methods are compared and some aspects of the role of *p*H are described

I want to thank Drs H Ettl, P Archibald, and H Bold for identifying the algae, Dr J v Gelder for isolating *Chlorococcum*, Dr Ph v Elteren for checking the statistical calculations, Dr G Barendse for correcting the English text, and Miss J Sterrenberg for technical assistance in part of the investigations Special thanks are due to Prof Dr H F Linskens who provided the opportunity for this study and stimulated it by his continued interest

TABLE 1. *Composition of media A-1 and A-2**

| | A-1 | A-2 |
|---|---|---|
| $KNO_3$ (mg/liter) | 500 | 200 |
| $K_2HPO_4$ (mg/liter) | 100 | 40 |
| $CaNO_3 \cdot 4H_2O$ (mg/liter) | 40 | 40 |
| $MgSO_4 \cdot 7H_2O$ (mg/liter) | 40 | 40 |
| KCl (mg/liter) | 10 | 10 |
| Fe-di-Na-EDTA (mg/liter) | 4 | 4 |
| $Na_2SiO_3 \cdot 9H_2O$ (mg/liter) | | 400 |
| Zn —as $ZnSO_4 \cdot 7H_2O$ ($\mu$g/liter) | 100 | 100 |
| Mn—as $MnSO_4 \cdot H_2O$ ($\mu$g/liter) | 75 | 75 |
| V —as $NH_4VO_3$ ($\mu$g/liter) | 25 | 25 |
| Co —as $CoCl_2 \cdot 6H_2O$ ($\mu$g/liter) | 25 | 25 |
| B —as $H_3BO_3$ ($\mu$g/liter) | 12.5 | 12.5 |
| Mo—as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ ($\mu$g/liter) | 10 | 10 |
| Cu—as $CuSO_4 \cdot 5H_2O$ ($\mu$g/liter) | 1 | 1 |
| $H_2SO_4$ (1.8%) (ml/liter) | 1 | |
| KOH (1.4%) (ml/liter) | 2 | |
| Difco Bacto-Agar (g/liter) | | 15 |

* Both media are prepared by mixing definite amounts from stock solutions of each compound (trace elements are added as one solution). To prevent the forming of a precipitate of calcium phosphate in A-1, the preparation is started with sulfuric acid. After all components have been added, and the volume is nearly made up to the desired value, potassium hydroxide is added to neutralize the excess sulfuric acid. All stock solutions and the media are prepared with deionized water. The *p*H of both media is about 7.

## MATERIALS AND METHODS

### *Species and culturing*

*Chlorococcum ellipsoideum* Deason and Bold (identified by Drs. P. Archibald and H. Bold, USA) and *Chlamydomonas globosa* Snow (identified by Dr. H. Ettl, Czechoslovakia), were isolated in 1967 from a little pond in the neighborhood of Nijmegen, "het Ketelven." The cultures were made axenic by a plating technique.

The culture medium was composed on the basis of data from Chu (1942), Rodhe (1948), and—with regard to micronutrients—Eyster (1964). The composition of the medium for liquid cultures (A-1) and the slightly modified A-2 for maintenance of the strains on agar slants is given in Table 1. In the experiments in which buffered medium was used, medium A-1 of double strength was mixed with an equal amount of Tris-HCl buffer of *p*H 8.1, which in turn was made by mixing equal amounts of Tris (0.2 M) and HCl (0.1 N).

Cultures were grown in a constant temperature room under a L : D cycle of 16 : 8 hr. The temperature was 25C in the light and 23C in the dark. Illumination was provided by batteries of fluorescent lamps (Philips TLM 40W/33RS and TLM 65W/33RS). The light intensity is given separately for each group of experiments. Tests for sterility were made by spreading a small amount of culture material on a plate of medium containing 15 g Difco Bacto-Agar, 14 g "Nahrbouillon" Merck, and 1 g Difco yeast extract in 1 liter of medium A-1. After 3 days of incubation at room temperature, plates were checked for bacteria and other microorganisms. Absence of colonies was taken as evidence that the algal culture from which the material came was axenic.

Inocula for the experiments were cultures in 50-ml erlenmeyer flasks with 10 ml of medium A-1. No shaking or aeration was necessary because of the great surface: volume ratio. In about 10 days such a culture attained maximum density, for inocula, 7-day cultures were used. Sterility tests were regularly made.

### *Design of experiments*

#### *Filter culture method*

The apparatus for this type of experiment is shown in Figs. 1 and 2. Glass cylinders (24 × 5.5 cm) were used as culture vessels. An outlet tube near the bottom of the cylinder served for aeration and sampling. Tubes for medium outlet and inlet and for air escape came through holes in the rubber stopper. An immersion filter unit was hung from the stopper, consisting of a fritted glass disk at the end of a glass funnel, onto which a Millipore HA filter (0.45-$\mu$ pore diam, 47-mm diam) was glued with a rubber-base glue. Every cylinder received 200 ml of medium A-1. A stirrer was immersed in the liquid below the filter unit, consisting of a magnetic bar built into a plastic-coated metal device with a soft brush of squirrel hair on top (this was meant originally to brush the cells from the filter to maintain a high flow rate, but the attempt was not successful and the devices were kept only as stirrers). All connections shown in Fig. 1

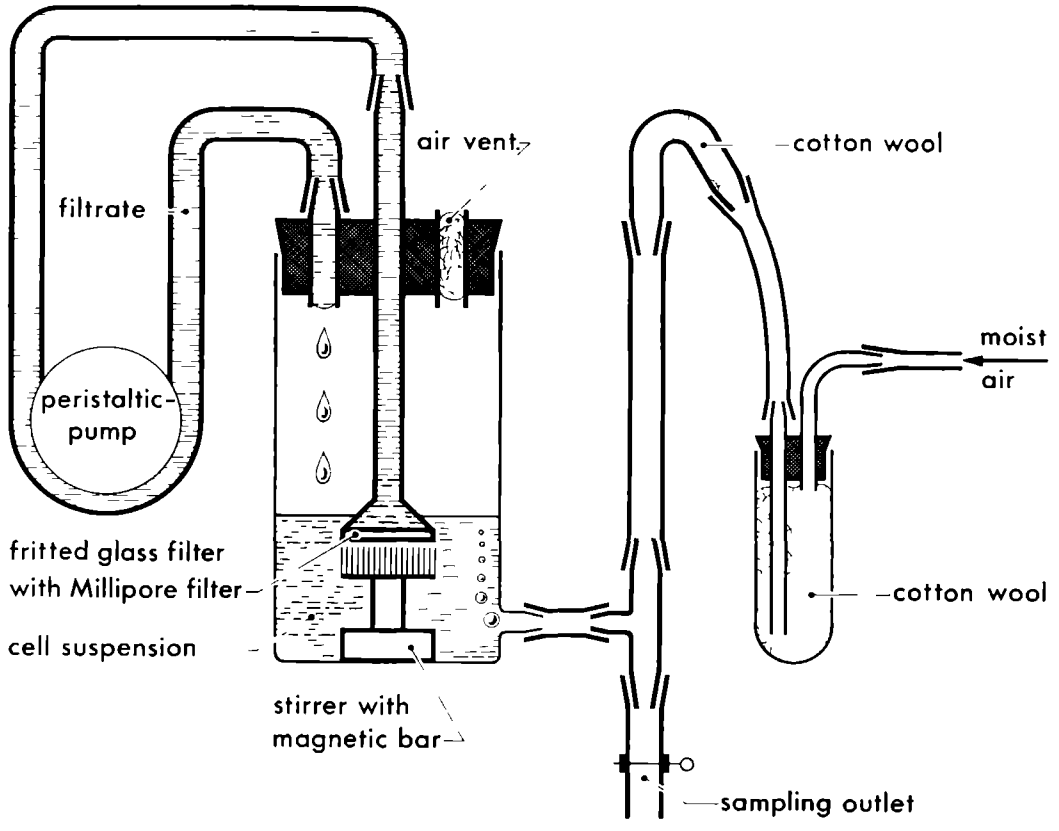



FIG 1. One unit in the assembly for filter culture experiments. Medium is filtered from the culture and pumped back by a peristaltic pump or pumped to another culture (cf. Fig. 2).

were made from silicone tubing, except for the tubing in the peristaltic pumps, which was Tygon. The whole assembly of four culture vessels with connections was autoclaved for 30 min at 110C. During autoclaving the filters were hung just above the liquid level, to avoid deformation of the Millipore membranes by the hot medium; after sterilization they were immersed about 5 mm deep in the medium.

The culture vessels were then inoculated: vessel A and B (Fig. 2) each with exactly equal amounts of *Chlorococcum* suspension (in most cases 5–10 ml), vessel C and D each with exactly equal amounts of *Chlamydomonas* suspension (0.3–0.5 ml). A and D constituted the controls, while B and C were the interconnected experimental cultures. Different amounts of inocula of the two species were necessary because *Chlorococcum* cultures had more cells adhering to the wall of the flask than in free suspension. The entire assembly was placed in the constant temperature room on magnetic stirrers, to keep the cells in suspension, at a light intensity of 7,500 lux. Air was led through the cultures after moistening in wash-bottles, via two cotton-wool filters (Fig. 1). After 2 days of culturing, when growth became visible, the connections with the peristaltic pump were made as seen in Fig. 2, to filter the medium. The levels in vessels B and C were kept about equal by hanging the filters just at the surface of the medium. The rate of flow through the filters was at first about 50 ml/hr, but after 1 day slowed down to about 20 ml/hr and then continued to decrease steadily, until at the end of the experiment (usually after 2.5 weeks) very little flow was left.

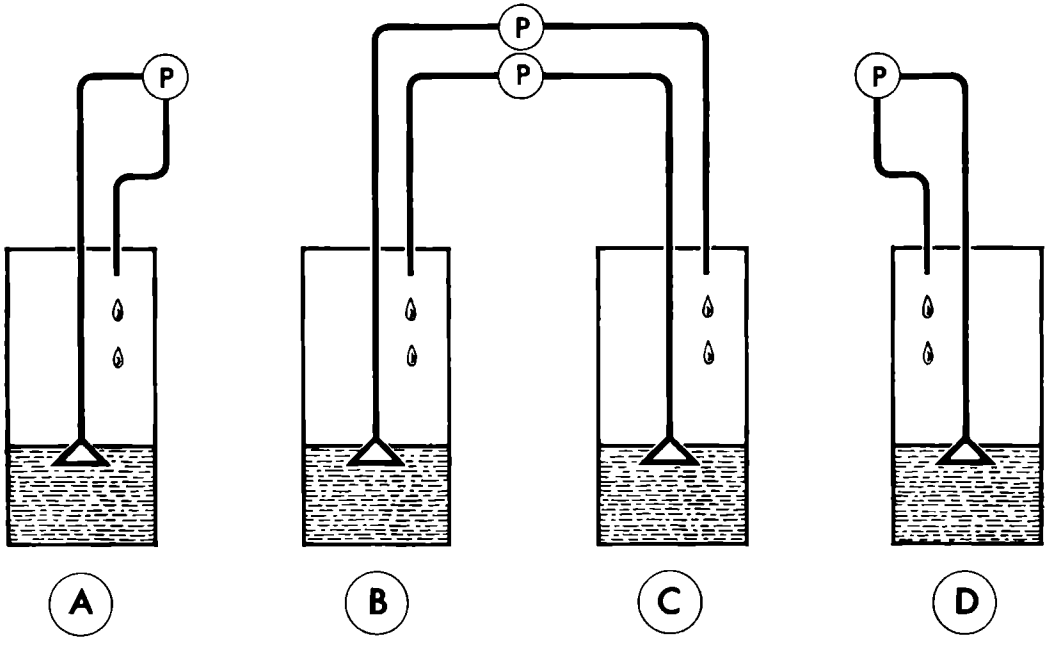



FIG. 2. Diagram of complete assembly in filter culture experiments. Compare Fig. 1 for details. A and D are the control cultures of *Chlamydomonas* and *Chlorococcum* respectively. B and C are the corresponding connected cultures. P = peristaltic pump (there is in fact only one, with several channels).

During culturing, 3-ml samples were drawn from each culture vessel every 2 or 3 days. The construction of the air inlet made it possible to use it also as a sampling outlet (Fig. 1); samples could be taken without risk of contaminating the culture, because there remained no liquid connection between the end of the outlet tube and the algal suspension (the air blowing the tube clean). However, the samples themselves were not sterile when taken by this method, so that sterility tests could not be made. Optical density of the samples was determined in a colorimeter with a 430-nm filter (transmission range from 420 to 465 nm) in a 0.5-cm cuvette, as a measure of the growth of the cultures (an O.D. of 0.1 corresponds roughly to a dry weight content of 250 mg/liter).

*Mixed culture method*

Small (50-ml) erlenmeyer flasks were used as culture vessels in these experiments. Each was provided with 10 ml of medium A-1 (buffered or unbuffered), closed with cotton plugs, and autoclaved for 20 min at 120C. A large number of cultures were started at the same time: a third inoculated with *Chlorococcum* (0.5-ml suspension), a third with *Chlamydomonas* (0.1-ml suspension), and a third with both 0.5 ml of *Chlorococcum* suspension and 0.1 ml of *Chlamydomonas* suspension. The cultures were placed in the constant temperature room at 4,500 lux (experiment with buffered medium) or 2,500 lux (experiment with unbuffered medium); they were not stirred, agitated, or aerated.

At regular intervals (usually every 2 days) several cultures (4 or 5 replicates for each treatment) were taken from the total group, always at the same time of day. A relative measure of cell density was obtained by extracting chlorophyll from each culture. The 10 ml of algal suspension was centrifuged, the supernatant used for *p*H measurement, and the residue, together with the algae remaining in the flask (adhering to the wall), extracted with 10 ml of 1 N KOH in methanol. The extract was cen-

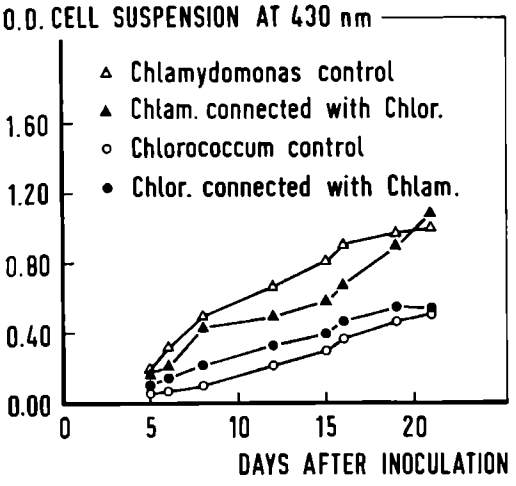



FIG. 3. Growth of *Chlamydomonas* and *Chlorococcum* in a filter culture experiment in unbuffered medium A-1. Each curve represents the growth of one culture.

trifuged and the O.D. of the clear supernatant was measured in the colorimeter at 430 nm (an O.D. of 0.1 corresponds roughly to a dry weight content of 100 mg/liter). The extraction was made with KOH in methanol because methanol alone only extracts total chlorophyll from *Chlamydomonas*, not from *Chlorococcum*; extraction took about 30 min at room temperature. Sterility of all cultures was checked as described on p. 871.

## RESULTS

The effect of *Chlamydomonas* and *Chlorococcum* on one another's growth was studied in two types of experiment.

### *Filter culture experiments*

The equipment used in these experiments was designed to culture two species in separate vessels while maintaining continuous mixing of the media from both cultures via filters. It proved to be difficult to keep the stirring and aeration rates the same for all four cultures of a set, and the results were somewhat variable. Clogging of the filters caused a rapid decrease of flow rate; particularly in the second half of each culture period mixing conditions were not ideal. This may also have contributed to the variability of the results. To obtain reliable data it was necessary to repeat the experiments many times. Altogether 13 experiments were carried out.

One of the resulting sets of growth curves is shown in Fig. 3. The curves do not have the well-known sigmoid shape nor did those in the other experiments. Possibly the limitation of air supply resulted in linear, rather than S-shaped growth of the populations. Moreover, after 2.5 weeks of culturing, the cells turned yellow, making O.D. measurements unreliable as a parameter of growth. In most experiments, therefore, growth could not be followed to maximum cell density. The problem was, how to evaluate the experiments statistically. Most of the 13 experiments were done seriatim, so that they could not be used as replicates. The following procedure was adopted: In each experiment, on each sampling date the difference in O.D. between the culture grown separately and that grown in contact with the other species was calculated, both for *Chlamydomonas* and *Chlorococcum*. Only the sign (+ or −) of the difference was recorded, not the magnitude. Differences of less than 0.003 O.D. units were regarded as nonexistent. For every experiment the total number of positive and negative differences was recorded for both species (Table 2).

A Wilcoxon sign rank test was carried out on these data, giving $P > 5\%$ for *Chlorococcum* and $P = 1\%$ for *Chlamydomonas*; the difference between controls and connected cultures was thus significant for *Chlamydomonas*, but not for *Chlorococcum*. From this, the preliminary conclusion may be drawn that, on the average, *Chlamydomonas* is inhibited by *Chlorococcum*, but *Chlorococcum* is apparently not affected by *Chlamydomonas*. The inhibition of *Chlamydomonas* results in an overall lower cell density during the experiment.

### *Mixed culture experiments*

It proved to be very difficult, with the two species investigated, to measure growth in mixed cultures for both species separately. Nevertheless, in some aspects this method had an advantage over the filter

TABLE 2 *Statistical evaluation of the filter culture experiments Wilcoxon's sign rank test*

| Exp No | No sampling dates with higher O D in: Separate culture | Combined culture | Difference |
|---|---|---|---|
| | Chlamydomonas* | | |
| 19 | 8 | 0 | +8 |
| 20 | 2 | 6 | −4 |
| 21 | 5 | 3 | +2 |
| 22 | 4 | 2 | +2 |
| 23 | 2 | 3 | −1 |
| 24 | 6 | 3 | +3 |
| 25 | 4 | 4 | 0 |
| 26 | 9 | 0 | +9 |
| 27 | 7 | 1 | +6 |
| 29 | 4 | 0 | +4 |
| 31 | 2 | 1 | +1 |
| 32 | 9 | 0 | +9 |
| 33a | 6 | 2 | +4 |
| 33b | 5 | 4 | +1 |
| | Chlorococcum* | | |
| 19 | 8 | 0 | +8 |
| 20 | 0 | 8 | −8 |
| 21 | 7 | 1 | +6 |
| 22 | 6 | 0 | +6 |
| 23 | 7 | 2 | +5 |
| 24 | 6 | 2 | +4 |
| 25 | 2 | 6 | −4 |
| 26 | 9 | 0 | +9 |
| 27 | 0 | 9 | −9 |
| 31 | 1 | 2 | −1 |
| 32 | 0 | 10 | −10 |
| 33a | 1 | 7 | −6 |
| 33b | 6 | 2 | +4 |

* For *Chlamydomonas* $T = 10$, $N = 13$, therefore $P = 1\%$ For *Chlorococcum* $T = 44$, $N = 13$, therefore $P > 5\%$

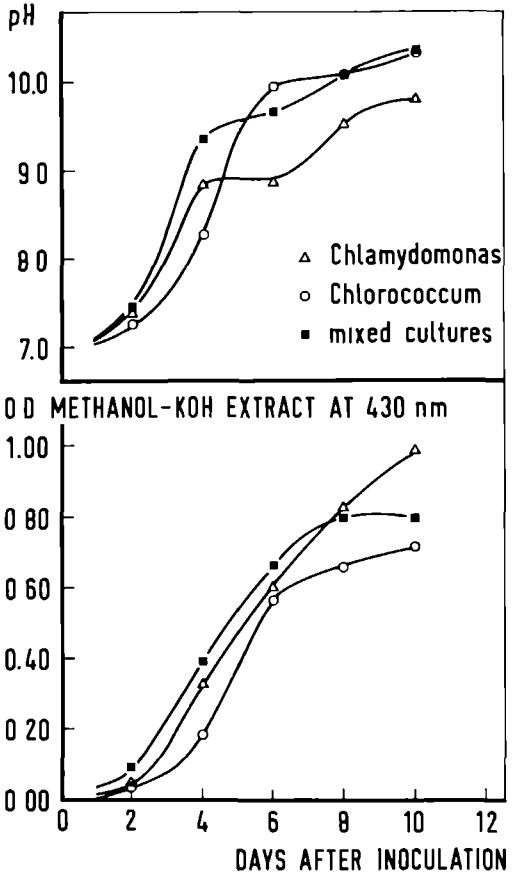



FIG 4 Growth of *Chlamydomonas* and *Chlorococcum* in mixed and separate cultures, in unbuffered medium A-1 Each value of O D and *p*H represents the mean from five cultures

culture method (a detailed comparison is given below), and several experiments were done in this way, but only total growth of the mixed cultures was measured. The results show that much information can be extracted from experiments of this kind, despite the absence of separate determination of the species.

In Fig 4 an experiment with unbuffered medium is shown. The mixed cultures closely follow the growth pattern of *Chlamydomonas* cultures during the first 6 days, thereafter—during the last 4 days—growth in the mixed cultures appears to be retarded appreciably. Since both *Chlamydomonas* and *Chlorococcum* are still growing separately, at least one of the two must be seriously inhibited in the mixed cultures The most likely interpretation of this is that *Chlamydomonas* first dominates in the mixed cultures by its faster start but later on is inhibited in growth or killed by *Chlorococcum* The sixth day is also the point where the *p*H of the *Chlorococcum* cultures reaches a shoulder after a very steep rise, and from that day on the *p*H in the mixed and the *Chlorococcum* cultures remain close together, indicating that it is mainly *Chlorococcum* that determines the *p*H of the mixed cultures at that stage. Repetitions of this experiment with different inoculation

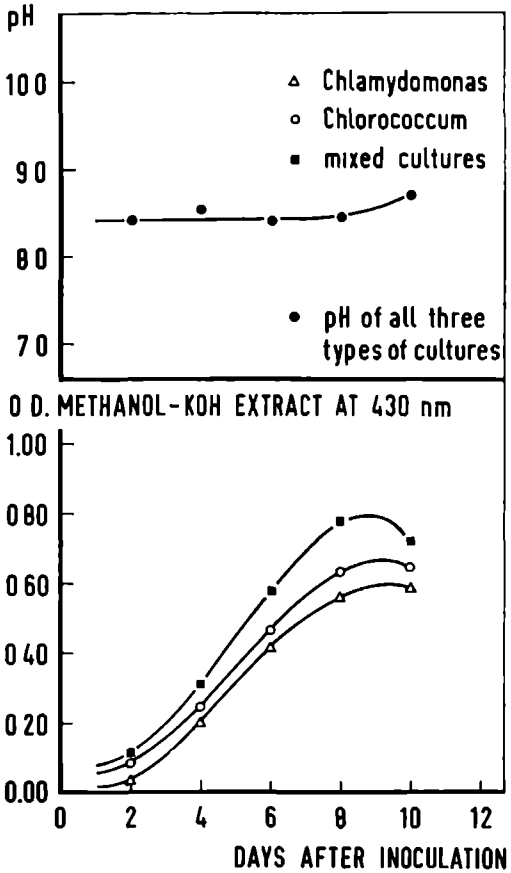



FIG 5 Growth of *Chlamydomonas* and *Chlorococcum* in mixed and separate cultures, in Tris-buffered medium A-1 Each value of O D and *p*H represents the mean from five (sometimes four) cultures The *p*H values were pooled because there was almost no difference between the three types of cultures

ratios (*Chlorococcum Chlamydomonas*) and different light intensities always gave essentially the same result, but a large inoculum of *Chlorococcum* caused an earlier decline of the mixed cultures

To determine whether *p*H affected the interaction of the species, experiments were repeated with medium buffered at *p*H 8 2 (Fig 5) Here the mixed cultures grew better than either *Chlamydomonas* or *Chlorococcum* separately Apparently one of the two, or both, species were stimulated by the presence of the other The *p*H remained almost constant at 8 2 for all cultures Es-

TABLE 3 *Statistical evaluation of the mixed culture (MC) experiments "Student's" t-test*

| Days after inoculation | Student's" *t* for difference* | | |
|---|---|---|---|
| | *Chlor* –MC | *Chlor* –*Chlam* | *Chlam* –MC |
| *Experiment with unbuffered medium (Fig 4)* | | | |
| 2 | 13 6 (8) | 7 1 (8) | 9 9 (8) |
| 4 | 10 1 (8) | 7 1 (8) | 2 9 (8) |
| 6 | 5 0 (8) | 2 4 (8) NS | 4 2 (8) |
| 8 | 7 1 (8) | 9 4 (8) | 2 5 (8) NS |
| 10 | 1 7 (8) NS | 6 3 (8) | 3 4 (8) |
| *Experiment with buffered medium (Fig 5)* | | | |
| 2 | 4 2 (8) | 11 0 (8) | 13 0 (8) |
| 4 | 10 7 (7) | 2 1 (8) NS | 5 0 (7) |
| 6 | 4 4 (7) | 1 3 (8) NS | 3 9 (7) |
| 8 | 3 9 (8) | 2 2 (8) NS | 7 8 (8) |
| 10 | 4 3 (6) | 2 7 (6) NS | 10 4 (6) |

* All differences are significant at the 1% level, except those marked NS Degrees of freedom in parentheses

sentially the same results were obtained from this experiment repeated at another light intensity.

The general conclusion to be drawn from both experiments shown is that *Chlamydomonas* is inhibited by *Chlorococcum* when the latter has the opportunity to change the *p*H of the medium, but that the species stimulate each other when the medium is buffered at *p*H 8 2 Apparently *Chlorococcum* is able to determine the *p*H once it is growing well in the mixed cultures *Chlamydomonas* seems to be sensitive to a *p*H somewhat higher than that which it reaches by itself in separate cultures (from day 4 on, the *p*H in the mixed cultures is at least half a unit higher than in the *Chlamydomonas* cultures) The question remains of course, whether this effect of *p*H is "direct," or if it acts, for example, by means of variations in salt availability It must be kept in mind that species were not determined separately in the mixed cultures, but in view of the results obtained with the filter culture method, the interpretation given here does not seem too farfetched

Rather than including statistical data in the graphs, I have presented the results of the calculation of "Student's" *t* for the differences between the groups of measurements in Table 3

## DISCUSSION

Interactions between aquatic microorganisms have been studied both in experiments using culture filtrates and in mixed culture experiments. The main advantage of using filtrates is that the growth of only one species needs to be measured. But it is almost impossible to test an unfractionated filtrate in such a way that the result is meaningful. Since the culture filtrate has necessarily been depleted of nutrient salts, growing a test organism on it will inevitably produce an inhibition effect that is without meaning for interaction processes. Competition for inorganic salts cannot be studied by this method, because it requires contact between the species over a certain period, while a filtrate represents only a momentary situation. Nor is it easy to discover inhibitory or promoting effects of extracellular substances, especially when only weak action is to be expected. To carry out an unequivocal test, it is necessary to make the filtrate up to the original composition of the salts, or to make a control medium of the same salt composition as the filtrates. Both possibilities are difficult to realize: No author in fact has tried. Lefèvre et al. (1952) and Proctor (1957) used unchanged filtrates as testing solutions. Jørgensen (1956) and Pratt (1966) added salts in the same amounts as present in fresh medium, so that the total concentrations became higher than in the original composition. Jakob (1957, 1961) worked with culture filtrates that were 20 times concentrated, but otherwise unchanged.

The mixed culture method has likewise been used many times, but only in the narrow sense of growing two species in the same culture vessel in the same medium. The filter culture method described here may be considered a variation on the mixed culture method sensu stricto; two species are essentially grown in the same medium (because of the exchange of medium via a filtering system), but not in the same vessel. In mixed cultures the situation seems to be more natural than in other experimental designs, but as in all natural systems it is difficult to separate different factors. In the case especially of two algae, it is impossible to separate physical factors (mainly light effects) from chemical interaction. Moreover, it is not always possible to measure growth of both species in a mixed culture separately. The only reliable method is to count the cells, either under the microscope, or—as Shindala et al. (1965) did with bacteria and yeasts—with the aid of an electronic cell counter. Both are useful only with organisms whose cells give homogeneous suspensions and are distinguishable in shape and size. These conditions were not fulfilled in the combination *Chlamydomonas-Chlorococcum* used here. *Chlorococcum* formed clusters of different sizes, and the species did not differ enough in size for electronic counter evaluation or in shape for fast and reliable microscopic counting.

The filter culture method has several advantages over the conventional mixed culture method: 1) light is excluded as a possible factor in interaction; only chemical processes can operate; 2) the species can be measured separately, by any method preferred (counting, weighing, chlorophyll determination, measuring O.D.); 3) the two species in the connected cultures are each growing in the same volume as their controls in the separate cultures, so that growth can be compared directly with that of the controls. Although there are still some practical difficulties with the method, especially that of keeping the filters clean to ensure a constant, rapid flow of filtrate, I believe that it will be possible to find a technical solution for this problem.

In the experiments described here, both methods provided information. The filter culture experiments showed that in unbuffered medium *Chlorococcum* inhibited the growth of *Chlamydomonas* by some chemical means, whereas *Chlamydomonas* did not have an influence on the growth of *Chlorococcum*. The mixed culture experiments with buffered and unbuffered medium gave a strong indication that the high *p*H produced by growing *Chlorococcum* was the reason (or at least one of the reasons) that *Chlamydomonas* grew less

well in combination with *Chlorococcum* than alone

In experiments of this kind, there is always the problem of the use of and the competition for nutrient salts If there had been serious differences in the rate of nutrient uptake, in nutrient preferences, or in growth rate, the "faster" species would always have grown at the cost of the slower" in the connected cultures Only a powerful toxic substance given off by the slower one might have counteracted this tendency, weak inhibiting (or promoting) actions would not have been noted Fortunately the two species grew at about the same rate in medium A-1, so it seems reasonable to assume that the observed inhibition of *Chlamydomonas* by *Chlorococcum* was not caused by competition for nutrient salts

The possibility of demonstrating an effect of *p*H in interaction experiments depends on the availability of suitable buffers *Chlamydomonas* and *Chlorococcum* grew excellently in Tris-buffered medium Preliminary experiments (Burgers, unpublished) had shown that the optimum *p*H for growth of both species was 8 0 to 8 5 To study the effect of higher *p*H values it will be necessary to search for other buffers with the same favorable characteristics as Tris, but in another *p*H range

The importance of the data obtained in this study for the understanding of species interactions in nature is difficult to assess The species used were from the same pond, but several other circumstances were less natural Only two species constituted the living part of the ecosystem, the concentration of nutrient salts was higher than in the pond, the *p*H was higher and perhaps more favorable for growth than in the natural water All these factors produced high cell densities Cells accumulated in microhabitats (e g , the bottom of shallow pools) could inhibit other species in the immediate vicinity of the algal layer (by a rise of *p*H, by excreted substances, or by other means) It is assumed that similar situations exist in soil by the interaction of microorganisms (e g , Brian 1957)

Almost no work has been done directly relating the findings of culture experiments to natural conditions Some workers have tried to test species of algae on water samples derived from natural sources especially from waterblooms (e g , Lefevre et al 1951, 1952) It is obviously difficult in such experiments to find the factors responsible for interaction, because of the unknown composition of the natural water Pratt (1966) related findings from culture experiments to observed periodic changes in phytoplankton composition in Narragansett Bay The compounds held responsible for the inhibition phenomena were not isolated or demonstrated to be present in the bay, although there were some indications from spectroscopy that they were present

## REFERENCES


Berglund, H 1969 Stimulation of growth of two marine green algae by organic substances excreted by *Enteromorpha linza* in unialgal and axenic cultures Physiol Plant **22** 1069–1073

Brian, P W 1957 The ecological significance of antibiotic production Symp Soc Gen Microbiol **7**: 168–188

Chu, S P 1942 The influence of the mineral composition of the medium on the growth of planktonic algae I J Ecol **30**: 284–325

Emeis, C -C 1956 Untersuchungen uber die antibakteriellen Eigenschaften der Algen Arch Hyg Bakteriol **140**: 597–604

Eyster, C 1964 Micronutrient requirements for green plants, especially algae, p 120–137 *In* D F Jackson [ed ], Algae and man Plenum

Gause, G F 1934 The struggle for existence Hafner (Reprint 1964 )

Goldeke, C G 1960 The ecology of a biotic community consisting of algae and bacteria Ecology **41**: 65–73

Hartman, R T 1960 Algae and metabolites of natural waters, p 38–49 *In* C A Tryon, Jr , and R T Hartman [eds ], The ecology of algae Univ Pittsburgh

Hobson, P N 1969 Growth of mixed cultures and their biological control Symp Soc Gen Microbiol **19**: 43–64

Hulburt, E M 1970 Competition for nutrients by marine phytoplankton in oceanic, coastal and estuarine regions Ecology **51** 475–484

Jakob, H 1954 Compatibilites et antagonismes entre algues du sol C R **238**: 928–930

——— 1957 Études sur certaines substances metaboliques liberees dans le milieu de culture par le *Nostoc muscorum* Ag C R **244**: 1968

——— 1961 Compatibilites, antagonismes et antibioses entre quelques algues du sol Rev Gen Bot **68**: 5–72

JØRGENSEN, E G 1956 Growth-inhibiting substances formed by algae Physiol Plant **9**: 712–726

———, AND E STEEMANN NIELSEN 1961 Effect of filtrates of unicellular algae on the growth of *Staphylococcus aureus* Physiol Plant **14**: 896–908

KAIN, J M, AND G E FOGG 1958 Studies on the growth of marine phytoplankton 1 J Mar Biol Ass U K **37**: 397–413

LANGE, W 1967 Effect of carbohydrates on the symbiotic growth of planktonic blue-green algae with bacteria Nature **215**: 1277–1278

LEFEVRE, M 1964 Extracellular products of algae, p 337–367 *In* D F Jackson [ed], Algae and man Plenum

——— H JAKOB, AND M NISBET 1951 Compatibilites et antagonismes entre algues d'eau douce dans les collections d'eau naturelles Int Ver Theor Angew Limnol Verh **11**: 224–229

——— ———, AND ——— 1952 Auto- et heteroantagonisme chez les algues d'eau douce —in vitro et dans les collections d'eau naturelles Ann Sta Cent Hydrobiol Appl **4**: 5–198

LEVINA, R J 1964 Interrelationships of various species of protococcal algae and their bactericidal effect in joint cultivation Microbiology (USSR) (Engl Transl) **33**: 120–126

MCVEIGH, I, AND W H BROWN 1954 In vitro growth of *Chlamydomonas chlamydogama* Bold and *Haematococcus pluvialis* Flotow em Wille in mixed cultures Bull Torrey Bot Club **81**: 218–233

MAKSIMOVA I V, AND E P FEDENKO 1965 The effect of the oxidation-reduction potential on the development of bacteria in algal cultures Microbiology (USSR) (Engl Transl) **34**: 286–290

———, AND M N PIMENOVA 1969 Influence of concomitant microflora on accumulation of organic compounds in medium during nonsterile culturing of *Chlorella* Microbiology (USSR) (Engl Transl) **38**: 509–513

NAKAMURA, H 1963 Studies on the eco system of *Chlorella*, p 197–204 *In* Studies on microalgae and photosynthetic bacteria Univ Tokyo

PRATT, D M 1966 Competition between *Skeletonema costatum* and *Olisthodiscus luteus* in Narragansett Bay and in culture Limnol Oceanogr **11**: 447–455

PROCTOR, V W 1957 Studies of algal antibiosis using *Haematococcus* and *Chlamydomonas* Limnol Oceanogr **2**: 125–139

PROVASOLI, L, AND I J PINTNER 1960 Artificial media for fresh-water algae problems and suggestions, p 84–96 *In* C A Tryon, Jr, and R T Hartman [eds], The ecology of algae Univ Pittsburgh

REICHART, G 1967 Die Synchronkultur von *Spirogyra* Ber Deut Bot Ges **80**: 177–186

RODHE, W 1948 Environmental requirements of fresh-water plankton algae Symb Bot Upsal **10**(1): 1–149

RUSCHMANN, G 1956 Beitrage zur Mikrobiologie der *Chlorella* 2 Biol Zentralbl **75**: 476–499

SILVDALA, A, H R BUNGAY, III, N R KRIEG, AND K CULBERT 1965 Mixed culture interactions 1 J Bacteriol **89**: 693–696

TALLING, J F 1957 The growth of two plankton diatoms in mixed cultures Physiol Plant **10**: 215–223

VELA, G R, AND C N GUERRA 1966 On the nature of mixed cultures of *Chlorella pyrenoidosa* TX 71105 and various bacteria J Gen Microbiol **42**: 123–131

VLADIMIROVA, M G 1961 Dynamics of the bacterial microflora growth in *Chlorella* cultures Microbiology (USSR) (Engl Transl) **30**: 374–377

———, AND L V BASAITOVA 1961 Development of *Chlorella pyrenoidosa* and bacteria of the *Pseudomonas* group on joint cultivation Microbiology (USSR) (Engl Transl) **30**: 500–505

# GROWTH INTERACTIONS BETWEEN *CHLAMYDOMONAS GLOBOSA* SNOW AND *CHLOROCOCCUM ELLIPSOIDEUM* DEASON AND BOLD: THE ROLE OF EXTRACELLULAR PRODUCTS

*H. W. Kroes*
Department of Botany, University of Nijmegen, Toernooiveld, Nijmegen/Netherlands

ABSTRACT

Extracellular substances were isolated from *Chlorococcum* culture filtrates and the four salt-free groups of substances obtained tested separately on *Chlamydomonas* growth. The group of steam-volatile substances had an overall promoting effect. The lipophilic group inhibited growth initially but had no lasting effect. The yellow, water-soluble pigments (phenolic compounds) could act as chelating substances The high molecular weight fraction (proteins and polysaccharides) promoted growth in the initial phase but had an inhibiting effect later on. These effects observed were mostly small but statistically significant

In algal interactions, inhibition phenomena are not likely to be caused by strong "antibiotic" substances. Factors such as *p*H may be more important than extracellular compounds as such.

## INTRODUCTION

In a previous paper (Kroes 1971) I described experimental ecosystems in which the effect of *Chlorococcum* and *Chlamydomonas* on each other's growth was studied. One conclusion was that *p*H played an important role in this interaction. Here, attention is given to extracellular compounds as possible factors in the relations between the two species.

Many compounds are known to occur in the culture filtrate of algae. Together, the following papers provide a rather complete survey of recent information: Weinmann (1970) (carbohydrates), Gocke (1970) (amino acids, polypeptides), Maksimova and Pimenova (1969) (organic acids), Collins and Kalnins (1965*a*, *b*) (volatile acids, alcohols, esters, carbonyl compounds), and Clémençon (1963, 1965) (B vitamins). A substance deserving of separate mention is glycolic acid, on which much work has been done (e.g. Lord et al. 1970). General reviews of the older literature have been given by Lefèvre (1964) and Fogg (1966). The total amount of carbon liberated into the culture medium by algae has been computed by Krogh et al. (1930) (5%), Hellebust (1965) (3–6%), Forsberg and Taube (1967) (2–5%), and Nalewajko and Marin (1969) (0.3–7.8%).

Sieburth (1968) and Whittaker and Feeny (1971) have reviewed the literature on the role of chemical substances in interactions between algal species. Reliable data are scarce although many workers have assumed the presence of specific "inhibitory substances" in cases where one species of algae was found to inhibit the growth of another (Kroes 1971). A recent example is Harris (1971). The neglect of factors such as competition for light, inorganic salts, etc., and *p*H, and the assumption that "antibiotic" substances were responsible for growth effects constitutes an oversimplification of the problem, since the inhibitors were not isolated in most investigations.

In the experiments described here, several groups of substances were isolated from the culture filtrate of *Chlorococcum*, freed from inorganic salts, and tested with regard to their effect on the growth of *Chlamydomonas*. Filtrates were always prepared from young, actively growing cultures to avoid the isolation of breakdown products from dead cells.

I want to thank Prof. H. F. Linskens for his continued support and interest, Miss J. Sterrenberg for accurate technical assistance, and Dr. G. Barendse for correcting the English text.

## MATERIAL AND METHODS

Details not mentioned here are given in Kroes (1971). Axenic cultures of *Chlamydomonas globosa* and *Chlorococcum ellipsoideum* were used throughout.

### *Preparation of* Chlorococcum *culture filtrates*

One-liter erlenmeyer flasks, each containing 400 ml of medium A-1, were autoclaved and inoculated with about 5 ml of a dense *Chlorococcum* suspension. The cultures were grown at about 24C at 7,000 lux under a L:D cycle of 16:8 hr. They were aerated with a mixture of 5% $CO_2$ and 95% air, but not shaken or stirred. Six days after inoculation the cultures, at about three-fourths their maximum density, were filtered through paper (Schleicher & Schüll, Selecta No. 595). Most of the cells remained on the filter or against the wall of the flask; zoospores were carried along in the filtrate, but cultures were filtered during the first hours of the dark period when a minimum of zoospores was present. A second filtration was done through Millipore HA (0.45-$\mu$ pore diameter) so that the final filtrate contained only dissolved substances; it was almost colorless.

### *Isolation of extracellular substances*

Except in the case of the substances of high molecular weight, the first step was always freeze-drying the filtrate. The solid residue was extracted successively with methanol and ethyl ether (respectively 50 and 25 ml for each liter of filtrate) at room temperature. Because the freeze-dried residue was finely dispersed, extraction could be completed in a few minutes. The combined methanol/ether extract contained most of the organic matter from the residue (except for the proteins and polysaccharides) while the greater part of the inorganic salts stayed behind. The distinctly yellow extract was evaporated under vacuum at 15C and the residue stored at -20C until further treatment.

*Steam-volatile substances*—The methanol /ether residue from 4,800 ml of filtrate was dissolved in 150 ml of water; the solution was acidified to *p*H 2 with $H_2SO_4$ and distilled at atmospheric pressure. A total of 100 ml of distillate was collected (the original solution was made up to 150 ml once during the process). The distillate had an acid odor.

*Lipophilic substances*—The methanol/ether extract was dissolved in 50 ml of water for every 2,400 ml of filtrate, resulting in a clear yellow-brown solution. It was acidified to *p*H 2 with $H_2SO_4$ and extracted with ether. The ether extract, containing the lipophilic substances, was washed with water, dried over anhydrous $Na_2SO_4$, and evaporated under vacuum at 15C, giving a yellow amorphous residue (= total lipid fraction). This was kept at -20C until the time of testing. From the water phase, retaining most of the original yellow color, the pigments were isolated (*see below*).

*Yellow-brown, water-soluble substances*—The water phase from the preceding extraction was freeze-dried; the solid residue was extracted with methanol at room temperature to dissolve the yellow-brown substances and to get rid of most of the inorganic salts. The methanol solution was evaporated under vacuum at 15C and the residue dissolved in 1–2 ml of 1 N KOH (water, or a weaker solution of KOH gave no completely clear solution at this high concentration of pigments). The dark-brown KOH solution was applied to a glass column filled with Sephadex G-10 (2 × 56 cm; bed volume 180 ml), and eluted with 0.01 N NaOH. Fractions of 5.5 ml were collected at a flow rate of 1 ml/min. The OD at 430 nm of the fractions was measured on a colorimeter and conductance after twofold dilution was measured on a conductivity meter with a cell constant of 1.25. Yellow fractions were detected visually during the check on elution volume; these were freeze-dried and kept at -20C until testing.

*High molecular weight substances*—For this the culture filtrate was not freeze-dried, but immediately ultrafiltered. The filtrate was forced through an Amicon Diaflo membrane, type PM-30, in an Amicon

ultrafiltration cell (model 202), under 1.5–3.5 $N_2$ pressure. This required about 20 hr at 4C for 1 liter of filtrate. The filtration was continued until about 20 ml of unfiltered solution was left over (= retentate). The retentate was made up to 200 ml with water and filtered again till 20 ml remained. This procedure was repeated twice. The final retentate thus contained only substances with a molecular weight of about 30,000 or more. It was sterilized by passing through Millipore HA filters and could in this state be stored at 4C until the time of testing. When the retentate was freeze-dried for weighing, a white fibrous coherent residue was obtained.

### *Testing of isolated substances*

The isolated fraction to be tested was dissolved in a nutrient solution that, in addition to the ingredients of medium A-1, contained tris(hydroxymethyl)aminomethane (Tris) 0.05 M and 0.025 N HCl, the *p*H of the cultures thus was held almost constant at 8.1 to 8.2. An identical nutrient solution, but without dissolved substances from *Chlorococcum* filtrates, was used as a control medium. Both the test and the control solutions were sterilized by passing through Millipore HA filters and dispensed aseptically in sterile 50-ml erlenmeyer flasks (10 ml of medium per flask) stoppered with cotton wool. Each flask was inoculated with 0.1 ml from a dense *Chlamydomonas* culture (dry wt, ca. 1 g/liter). The cultures were grown at about 24C at 4,500–6,000 lux under a L:D cycle of 16:8 hr. They were not aerated, stirred, or shaken. Measurements of growth and *p*H and sterility tests were made every 2 or 3 days by taking four or (usually) five cultures from the test and control groups, respectively, and processing each culture as follows. The 10 ml of algal suspension was centrifuged; the supernatant was used for *p*H determination and the residue, together with the cells still adhering to the wall of the culture flask, was extracted with 10 ml of methanol. The extract was centrifuged and the OD at 430 nm of the clear supernatant measured in a 0.5-cm cuvette. The relation between OD at this wavelength and dry weight of the cells was linear, an OD value of 0.1 corresponding roughly with a dry weight of 90 mg/liter. The growth curves were constructed by computing the mean of the OD values for each day of measurements; each growth curve therefore represents the average growth of a group of cultures, not the average of growth curves of individual cultures.

Where necessary, a "Student's" *t*-test was used to detect statistically significant differences between means of test and control cultures. The *p*H of the cultures during growth is not mentioned explicitly for all points because it always remained close to 8.1 between limits of at most 0.2 units; during growth it showed a slight tendency to rise in all experiments.

### *Culture purity*

Purity of both the *Chlorococcum* and the *Chlamydomonas* cultures was tested by spreading a droplet of cell suspension over a B-2 agar plate (B-2: 1.0 g each of glucose, Nahrbouillon Merck, and Difco yeast extract, together with 15 g of Difco bacto-agar dissolved in 1 liter of medium A-1). After 4 days of incubation at 24C, plates were checked for contaminants. In the experiments reported below only a few cases of contamination occurred (<5% of the cultures). Contaminated *Chlorococcum* cultures were not used for filtrate preparation.

### *Chemical identification and quantitative determination of isolated groups of compounds*

All purified fractions were weighed, in most cases starting from 4,800 ml of filtrate or more. Estimates of content in the original filtrate are probably too low because of losses during the purification procedure. Protein content of the high molecular weight fraction was measured according to the method of Lowry et al. (1951) with bovine albumin as a standard. Carbohydrate content of the same fraction

was measured according to the anthron method of Hewitt (1958) with glucose as a standard. Spot tests for the presence of phenolic compounds in the yellow-brown pigment fraction were: 1) Le Rosen test (on aromatic compounds) and 2) Ehrlich diazo test (on phenol and imidazole derivatives), both as described in Feigl (1966); 3) tetrazotized benzidine test (on phenols) and 4) $FeCl_3$ test (on phenols), both modified for spot test after Christman and Ghassemi (1966). An absorption spectrum of the yellow-brown pigments in methanol : water (5 : 1) was measured on a Zeiss PMQ II spectrophotometer. A fatty acid determination according to Heinen and de Vries (1966) was carried out on the lipophilic fraction with lauric and myristic acid as standards.

## RESULTS

It proved necessary to test all compounds in a concentration 10–25 times that in the original filtrate; lower concentrations gave growth effects too small to be detected statistically. A possible effect of variations in *p*H (as demonstrated in former experiments: Kroes 1971) was excluded by the use of Tris-buffered medium.

### *Steam-volatile substances*

Because the filtrate had been freeze-dried under high vacuum (<0.1 mm of Hg) and, after that, the methanol/ether extract had been evaporated under vacuum, it is not probable that appreciable amounts of volatile substances were left over other than carboxylic acids, which at the *p*H of the filtrate (about 8.0) were present as their potassium salts. These became volatile only after liberation of the original acids by acidification. Titration with NaOH showed that the distillate was about $3.10^{-4}$ N in acid. For testing, the distillate was mixed with the required amounts of A-1, Tris, and HCl to give a medium of the right composition (*see methods*). The control medium was made by replacing the acid distillate by a distillate from deionized water.

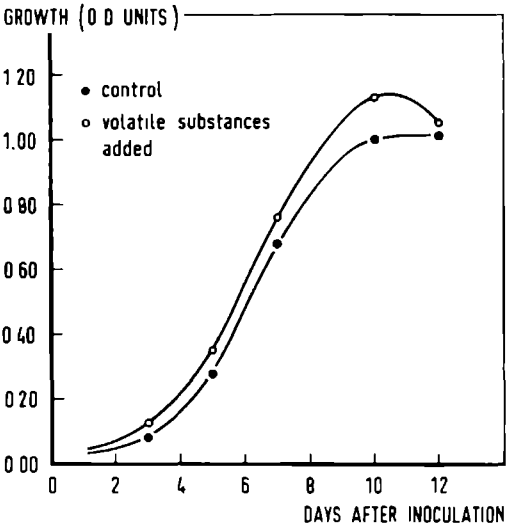



FIG. 1. Growth of *Chlamydomonas* in medium supplemented with the steam-volatile substances from *Chlorococcum* culture filtrate; *p*H from 8.0 (beginning) to 8.2 (end) in both control and test cultures. In this and the following figures the parameter of growth is the OD at 430 nm of a methanol extract of each culture. Each value is the mean of data from five cultures.

The growth of *Chlamydomonas*—both the initial growth and the finally attained maximum—was promoted by the distillate (Fig. 1). Statistically significant differences showed on days 3, 10, and 12 ($P < 5\%$). The concentration factor of the isolated compounds here was about 8; the experiment was repeated with essentially the same results.

### *Lipophilic substances*

Their behavior in the extractions carried out characterizes the lipophilic substances as organic acids, soluble in water in the salt form but insoluble in the free acid form. A large proportion of them could be extracted from ether by 10% $NaHCO_3$, an indication of the presence of carboxylic acids up to $C_{12}$ (lauric acid) (Katayama 1962). The weight of the total lipid fraction was computed to be about 5 mg/liter in the original filtrate. A fatty acid determination according to Heinen and de Vries (1966) showed that 10%

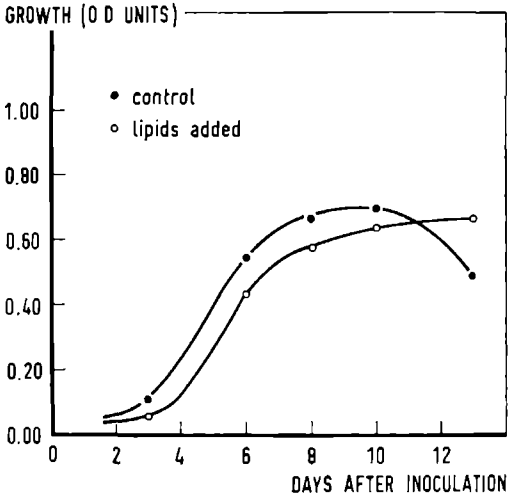



FIG. 2. Growth of *Chlamydomonas* in medium supplemented with the lipophilic substances from *Chlorococcum* culture filtrate; *p*H from 8.9 (beginning) to 9.0 (end) in both control and test cultures. Each value is the mean of data from five cultures.

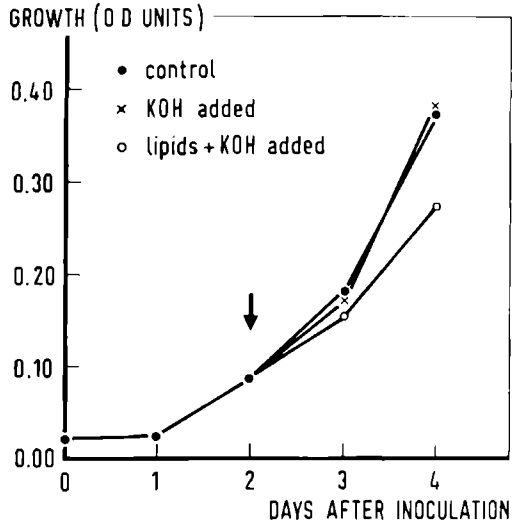



FIG. 3. Growth of *Chlamydomonas* in medium supplemented with the lipophilic substances from *Chlorococcum* culture filtrate; addition of the compounds only after 2 days of growth (arrow); *p*H from 8.1 (beginning) to 8.2 (end) for all three treatments. Each value is the mean of data from five cultures.

of the lipophilic substances were normal fatty acids from $C_{12}$ through $C_{18}$.

The total lipid fraction was tested on *Chlamydomonas* by dissolving the purified residue in 5 ml of 1 N NaOH and adding this solution to a nutrient medium with excess HCl to compensate for the NaOH added; for a control, an identical nutrient medium was mixed with 5 ml of 1 N NaOH. The concentration factor in this experiment was about 23. The initial growth rate of *Chlamydomonas* is distinctly lower with than without the lipids. The maximum density, however, is almost unaffected by the lipids (Fig. 2). The experiment was repeated with essentially the same results.

An additional experiment was done in which the lipids were added only after 2 days of growth; in this case a double control was provided by leaving a third of the cultures unchanged, adding 1 ml of 0.017 N KOH to each flask of another third of the cultures, and adding 1 ml of 0.017 N KOH containing the lipids to each flask of the last third. In this experiment the concentration factor of the lipids was about 20. Figure 3 (the second experiment) gives as it were a magnification of the first part of the growth. Clearly, growth does not stop, but is only retarded on the first and second days after addition of the lipids. Addition of KOH above apparently does not interfere with growth. Statistically significant differences in Fig. 2 are on days 3, 6, 8, and 13 ($P < 5\%$); in Fig. 3 on days 3 (between "control" and "lipids + KOH added") and 4 (between "control" and "lipids + KOH added," and between "KOH added" and "lipids + KOH added") ($P < 1\%$).

### *Yellow, water-soluble pigments*

The result of chromatography of the partially purified yellow pigments is shown in Fig. 4. The pigments were eluted very rapidly (peak in OD). Salts and other electrolytes were eluted afterwards (peaks in conductance). Therefore, it may be assumed that the fractions containing the OD peak were free of inorganic salts and of organic substances of low molecular

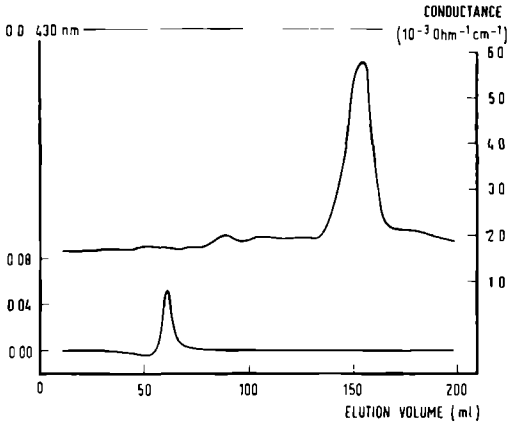



FIG. 4. Separation of the yellow, water-soluble pigments from salts and other impurities on Sephadex G-10; peak in OD (lower curve) represents fractions with yellow compounds; peaks in conductance (upper curve) represent salts and other electrolytes.

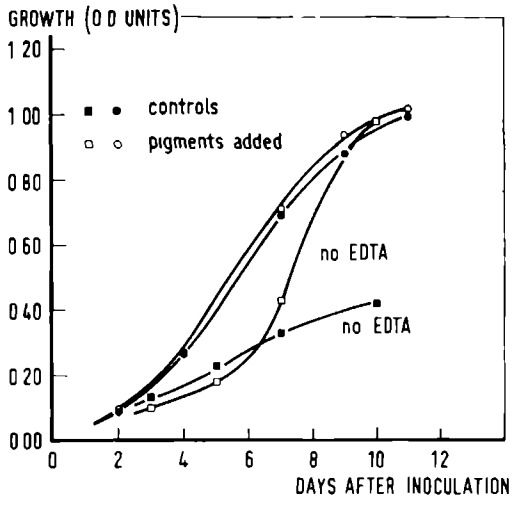



FIG. 5. Growth of *Chlamydomonas* in medium with and without EDTA supplemented with the yellow, water-soluble pigments from *Chlorococcum* culture filtrate; *p*H from 8.1 (beginning) to 8.2 (end) in both control and test cultures. Each value is the mean of data from five cultures.

weight (e.g. amino acids). The elution time of the pigments indicates a molecular weight of >1,000; the upper limit is <30,000, because they passed the PM-30 membrane easily (*see* p. αβ). Since I suspected that the pigments might be phenolic, some spot tests were carried out. The $FeCl_3$ test and the tetrazotized benzidine test were positive, but the Le Rosen test and the Ehrlich diazo test were negative. When applied to paper as a spot, the pigments showed a blue fluorescence in ultraviolet light of 254 nm. An absorption spectrum from 230–700 nm gave a curve without peaks, descending smoothly from a high absorbance at 230 nm to zero absorbance at 700 nm, with a slight shoulder at about 300 nm. The weight of the pigments was determined by freeze-drying the two or three fractions containing the yellow peak and, as a reference, the same number of fractions immediately preceding the yellow ones. Because both contained the same amount of NaOH from the elution liquid, the difference in weight was the weight of the yellow pigments. There appeared to be about 4 mg /liter in the original filtrate.

For testing, freeze-dried yellow Sephadex fractions were dissolved in nutrient medium, with the same number of fractions immediately preceding the yellow ones used as a control. Both groups of fractions contained the same amount of NaOH. Two types of experiment were done: one as described under methods, and a second one with a nutrient solution in which Fe-di-Na-EDTA was replaced by an Fe equivalent amount of $FeSO_4 \cdot 7H_2O$. The concentration factor in the first experiment was 29, in the second 24. Figure 5 shows the effect of the pigments. Growth of *Chlamydomonas* in normal medium is promoted very slightly, not significantly different from the control. When the chelating substance EDTA is omitted, however, growth is inhibited strongly in the controls but scarcely so in the cultures with added pigments. Because standard deviations were very high in this experiment, a statistically significant difference was found only on day 10 ($P < 1\%$). The experiments were repeated with essentially the same results.

### *High molecular weight substances*

The substances retained by the PM-30 membrane must have had a molecular weight of 30,000 or more. For polysaccharides this value is even higher: the retention characteristic of the membrane depends not only on molecular weight, but also on the shape and chemical nature of the substances concerned. A protein determination on this fraction yielded about 4 mg bovine albumin equivalent per liter of original filtrate; a carbohydrate determination yielded about 4 mg glucose equivalent per liter of filtrate. The high molecular weight fraction may have consisted of polysaccharides and proteins separately or of a glycoprotein giving a positive reaction to both protein and carbohydrate tests. Direct weighing of the whole freeze-dried fraction resulted in an estimate of about 11 mg/liter in the original filtrate.

The effect of the whole high molecular weight fraction on *Chlamydomonas* was tested by mixing the sterilized solution (*see methods*) with the right amounts of A-1, Tris, and HCl and comparing growth in this nutrient medium with that in a control medium containing water instead of the protein/polysaccharide solution. The high molecular weight substances stimulate the growth of *Chlamydomonas* during the first days after inoculation but have a negative effect on the maximum density reached (Fig. 6). Statistically significant differences are on days 5 and 12 ($P < 0.1\%$). Repetition of the experiments gave essentially the same results.

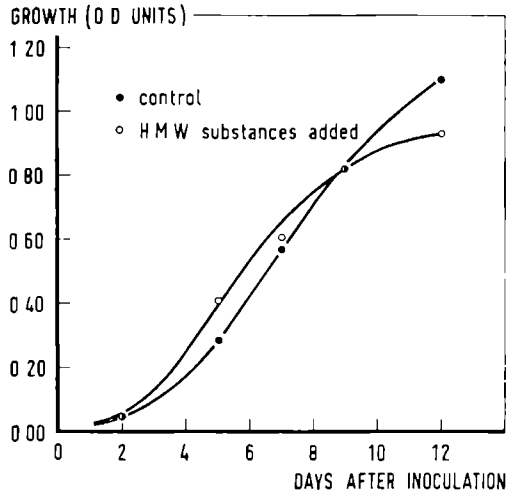



FIG. 6. Growth of *Chlamydomonas* in medium supplemented with the fraction of high molecular weight substances (HMW) from *Chlorococcum* culture filtrate, *p*H from 8.2 (beginning) to 8.3 (end) in both control and test cultures. Each value is the mean of data from five cultures.

### *The effect of known fatty acids on the growth of* Chlamydomonas

Because fatty acids were detected in the lipophilic fraction, some experiments were done to find out whether these could be responsible for the observed growth inhibition. Tests were similar to those described for the isolated substances, but only one point of the growth curve was measured. Fatty acids (sigma) were dissolved in part of the Tris needed to make the nutrient solution, and then the other ingredients were added. The control was a normal nutrient medium. Capric acid ($C_{10}$), lauric acid ($C_{12}$), and myristic acid ($C_{14}$) each inhibited the growth of *Chlamydomonas* in a concentration of 5 mg /liter. For example, in the presence of $C_{10}$ five cultures developed a mean OD of 0.067 after 4 days of growth (controls: 0.157); $C_{12}$ gave an OD of 0.023 after 5 days of growth (controls: 0.219); $C_{14}$ gave an OD of 0.130 after 5 days of growth (controls: 0.168). The *p*H of the cultures was 8.1–8.2. For all three acids the differences in OD between test and control cultures were significant ($P < 0.1\%$).

## DISCUSSION

Several workers have investigated growth interactions in algae (*see* Kroes 1971), but almost no experiments have been done in which defined groups of substances were isolated from healthy, axenic cultures and tested on other species. This type of testing has the disadvantage that the total effect of extracellular substances in the filtrate cannot be studied, but there is

ample compensation in the absence of inorganic salts which might interfere with the tests and in the fact that the effect of each group of substances can be determined separately. These conditions cannot be realized in whole-filtrate testing—the commonly used method.

In the experiments here described care was taken not to damage the *Chlorococcum* cells (e.g. by shaking or stirring) and to prepare filtrates from actively growing cultures only; liberation of substances from dead cells was thus avoided. There are three possible sources of organic substances in an algal culture medium: "leakage" from living cells (whatever its mechanism); dissolving of substances from cell walls and protoplasmic remnants resulting from cell divisions; liberation of material from dead cells. In favor of leakage is the well-documented case of glycolic acid, where excretion from living cells has been demonstrated beyond doubt (e.g. Lord et al. 1970) and also the fact that some workers found highest excretion values during the phase of fastest growth (Nalewajko and Marin 1969; Watt 1969). In contrast, Marker (1965) and Maksimova et al. (1965) found high values for extracellular substances in the period immediately after inoculation; they supposed that damage to cells and liberation of products during cell division, respectively, were responsible.

For the evaluation of the effect of isolated substances various parameters can be measured, such as cell density after a certain period of growth, or growth rate during the exponential phase. I have determined the whole growth curves of the cultures, because this allows a more detailed analysis of the effect than the other methods.

The steam-volatile substances distinctly promoted the growth of *Chlamydomonas*. This is easy to understand since they were mainly low molecular weight carboxylic acids. It is known that acetate, for example, can serve as an organic carbon source for unicellular algae. The mechanism of release of the acids into the nutrient medium is probably leakage from living *Chlorococcum* cells.

The lipophilic fraction (probably fatty acids in the range of $C_8$ to $C_{18}$ and phenolic acids) had an inhibiting effect on *Chlamydomonas* expressed in a retardation of growth during the first days after the addition of the lipids. The cause of this inhibition may be the surface activity of the fatty acids and their salts. The disappearance of the inhibition in the course of growth may result from the interaction of excretion products of *Chlamydomonas* with the added compounds. The experiments with known fatty acids showed that concentrations of a few milligrams per liter could severely inhibit the growth of *Chlamydomonas* in the phase after inoculation, so the inhibition caused by the *Chlorococcum* lipids may be due to fatty acids. It does not seem probable that fatty acids are easily excreted from the cells; a more plausible source is the cell walls—both of living cells and of division remnants. Lipids do occur in algal cell walls (Northcote et al. 1958; Pakhomova and Zaitseva 1969). The yellow, water-soluble substances found in the *Chlorococcum* filtrate may be related to the yellow, mostly unidentified compounds found in seawater ("Gelbstoff": Kalle 1966; Sieburth and Jensen 1968), in freshwater (Christman and Ghassemi 1966), and in axenic cultures of brown algae (Fogg and Boalch 1958). None of them has been exactly identified, nor is it certain that they are closely related; but there are indications that they all are phenolic compounds.

The fact that the yellow pigments had almost no effect on the initial growth rate or on the final density of *Chlamydomonas* suggests that they do not serve as an organic substrate. When yellow pigments were added to EDTA-lacking cultures, they had a very favorable effect on growth, suggesting that they may act as chelating substances in keeping iron and other elements available to the algae. Fulvic acid, a water-soluble fraction of humic material, had a promoting effect on the

growth of some blue-green algae by its chelating properties (Lange 1970).

The peculiar effect of the high molecular weight fraction on the growth of *Chlamydomonas*—a stimulation of the initial growth rate, followed by a reduction of the maximum density reached—is hard to explain. Perhaps partial hydrolysis of the polysaccharides liberates sugars, promoting initial growth, and the inhibiting effect later may be the consequence of the binding of essential metal ions by the proteins. The polysaccharides and proteins in the *Chlorococcum* filtrate probably both come from the cell walls (Northcote et al. 1958; Thompson and Preston 1967, Pakhomova and Zaitseva 1969).

One may ask whether it makes sense from an ecological point of view to test those substances in such high concentrations. The experiments were not intended to yield results directly applicable to the natural situation; they were meant to find out what kind of interactions are possible between algal species and to work out reliable methods for doing this, and it proved necessary to test the isolated substances in much higher concentrations than those in the filtrate because otherwise the effects would have been too small to detect. Although in the *Chlorococcum* filtrate the compounds were present in amounts of 5–10 mg/liter, the concentrations in the tests were in the order of magnitude of 100 mg /liter. In natural waters the concentration of organic compounds seldom approaches this value (e.g. Wagner 1969: 1–2 mg/liter in seawater, Gocke 1970: 4–15 mg/liter in freshwater). However, in the immediate neighborhood of algal concentrations in nature (layers on the bottom of shallow waters, on aquatic macrophytes, etc.) there will be a buildup of liberated organic matter in solution, especially when turbulence is low. Concentrations of these compounds may thus be many times higher locally than in a body of water as a whole.

The most important facts found here are that there are many different extracellular compounds in cultures of *Chlorococcum*—which is not surprising—and that each fraction appears to have its own specific but small effect on the growth of *Chlamydomonas*. This picture is different from that suggested by other authors (*see* Kroes 1971) who have generally assumed that, in the algal ecosystem they were studying, rather strong inhibitory substances were responsible for the observed inhibition phenomena. On the whole, it seems that the role of *p*H, at least in the range of 9–11 (Kroes 1971), is more important in interactions between *Chlorococcum* and *Chlamydomonas* than that of extracellular substances.

## REFERENCES


CHRISTMAN, R. F., AND M GHASSEMI 1966 Chemical nature of organic color in water. J Amer Water Works Ass **58**: 723–741

CLÉMENÇON, H. 1963 Verbreitung einiger B-Vitamine in Kleingewassern aus der Umgebung von Bern und Untersuchungen uber die Abgabe von B-Vitaminen durch Algen Schweiz Z Hydrobiol. **25**: 157–165

——— 1965 Untersuchungen zur Folsaure-Ausscheidung durch *Nannochloris schopferi* Pringsheim und *Chlorocloster engadinensis* Vischer Arch Mikrobiol. **51**: 213–232.

COLLINS, R P, AND K KALNINS 1965*a* Volatile constituents of *Synura petersenii* 1. Lloydia **28**: 48–52.

———, AND ——— 1965*b* Volatile constituents produced by the alga *Synura petersenii* 2. Int J Air Water Pollut **9**: 501–504

FEIGL, F. 1966 Spot tests in organic analysis, 7th English ed Elsevier

FOGG, G E 1966 The extracellular products of algae Oceanogr. Mar Biol Annu Rev **4**: 195–212

———, AND G. T. BOALCH 1958 Extracellular products in pure cultures of a brown alga Nature **181**: 789–790

FORSBERG, C, AND O TAUBE 1967 Extracellular organic carbon from some green algae Physiol Plant **20**: 200–207.

GOCKE, K. 1970 Untersuchungen uber Abgabe und Aufnahme von Aminosauren und Polypeptiden durch Planktonorganismen. Arch Hydrobiol **67**: 285–367

HARRIS, D. O 1971 Growth inhibitors produced by the green algae (Volvocaceae) Arch Mikrobiol. **76**: 47–50

HEINEN, W., AND H DE VRIES. 1966 A combined micro- and semi-micro colorimetric determination of long chain fatty acids from plant cutin. Arch. Mikrobiol **54**: 339–349

HELLEBUST, J A. 1965. Excretion of some organic compounds by marine phytoplankton. Limnol. Oceanogr. **10**: 192–206.

HEWITT, B R 1958 Spectrophotometric determination of total carbohydrate Nature **182**: 246–247

KALLE, K 1966 The problem of the Gelbstoff in the sea Oceanogr Mar Biol Annu Rev **4**: 91–104

KATAYAMA, T 1962 Volatile constituents, p 467–473 *In* R A Lewin [ed ], Physiology and biochemistry of algae Academic

KROES, H W 1971 Growth interactions between *Chlamydomonas globosa* Snow and *Chlorococcum ellipsoideum* Deason and Bold under different experimental conditions, with special attention to the role of *p*H Limnol Oceanogr **16**: 869–879

KROGH, A , E LANGE, AND W SMITH 1930 On the organic matter given off by algae Biochem J **24**: 1666–1671

LANGE, W 1970 Blue-green algae and humic substances Proc 13th Conf Great Lakes Res (Int Ass Great Lakes Res ) **1970**: 58–70

LEFEVRE, M 1964 Extracellular products of algae, p 337–367 *In* D F Jackson [ed ], Algae and man Plenum

LORD, J M , G A CODD, AND M J MERRET 1970 The effect of light quality on glycolate formation and excretion in algae Plant Physiol **46**: 855–856

LOWRY, O H , N J ROSEBROUGH, A L FARR, AND R J RANDALL 1951 Protein measurement with the Folin phenol reagent J Biol Chem **193**: 265–275

MAKSIMOVA, I V , AND M N PIMENOVA 1969 Liberation of organic acids by green unicellular algae Microbiology (USSR) (Engl Transl ) **38**: 64–70

———, E C TOROPOVA, AND M N PIMENOVA 1965 Secretion of organic substances in the growth of green algae on mineral media Microbiology (USSR) (Engl Transl ) **34**: 413–419

MARKER, A F H 1965 Extracellular carbohydrate liberation in the flagellates *Isochrysis galbana* and *Prymnesium parvum* J Mar Biol Ass U K **45**: 755–772

NALEWAJKO, C , AND L MARIN 1969 Extracellular production in relation to growth of four planktonic algae and of phytoplankton populations from Lake Ontario Can J Bot **47**: 405–413

NORTHCOTE, D H , K J GOULDING, AND R W HORNE 1958 The chemical composition and structure of the cell wall of *Chlorella pyrenoidosa* Biochem J **70**: 391–397

PAKHOMOVA, M V , AND G N ZAITSEVA 1969 Study of the chemical composition of the cell wall of the unicellular green alga *Chlamydomonas globosa* Microbiology (USSR) (Engl Transl ) **38**: 575–578

SIEBURTH, J MCN 1968 The influence of algal antibiosis on the ecology of marine microorganisms, p 63–94 *In* M R Droop and E J Ferguson Wood [eds ], Advances in microbiology of the sea, v 1 Academic

———, AND A JENSEN 1968 Studies on algal substances in the sea 1 J Exp Mar Biol Ecol **2**: 174–189

THOMPSON, E W , AND R D PRESTON 1967 Proteins in the cell walls of some green algae (*Nitella opaca, Cladophora Chaetomorpha, Codium*) Nature **213**: 684–685

WAGNER, F S , JR 1969 Composition of the dissolved organic compounds in seawater a review Contrib Mar Sci **14**: 115–153

WATT, W D 1969 Extracellular release of organic matter from two freshwater diatoms Ann Bot **33**: 427–437

WEINMANN, C 1970 Geloste Kohlenhydrate und andere organische Stoffe in naturlichen Gewassern und in Kulturen von *Scenedesmus quadricauda* Arch Hydrobiol Suppl 37, p 164–242

WHITTAKER, R H , AND P P FEENY 1971 Allelochemics chemical interactions between species Science **171**: 757–770

Arch. Mikrobiol. 84, 270—274 (1972)


# Extracellular Products from *Chlorococcum ellipsoideum* and *Chlamydomonas globosa*

H. W. Kroes

Department of Botany, University of Nijmegen, The Netherlands

Received February 7, 1972

*Summary.* Extracellular products of *Chlorococcum* and *Chlamydomonas* were isolated from culture filtrates. Five groups of substances were found in both species: (i) steam-volatile acids, (ii) yellow, water-soluble phenolic compounds, (iii) lipophilic substances, (iv) proteins, and (v) polysaccharides. *Chlorococcum* had higher concentrations of all groups than *Chlamydomonas*; moreover, the two species differed slightly in the composition of groups (ii) and (iii). Probably, there is a great similarity between green algae in the production of extracellular compounds.

In spite of a growing interest in the extracellular products of algae during the last two decennia, not much is known about the diversity of substances which are liberated into the surrounding medium by a single species. Most studies (references: see Kroes, 1972) include only one group of substances, e.g. carbohydrates, organic acids, etc. In the course of an investigation on interactions between two fresh-water algae under laboratory conditions (Kroes, 1971, 1972) the effect of extracellular substances from *Chlorococcum* on the growth of *Chlamydomonas* was studied and their ecological importance was discussed. The question arose if the compounds were typical of *Chlorococcum* and if *Chlamydomonas* – not closely related to *Chlorococcum* – would produce the same or another array of substances. Therefore, a comparison was made of the extracellular products of the two species.

## Materials and Methods

Details not mentioned here have been described in Kroes (1971, 1972).

*Culture Filtrates. Chlorococcum* filtrates were prepared from six days old cultures (dry weight of cells 0.5 g/l); the cultures were filtered through paper and Millipore HA, respectively. *Chlamydomonas* filtrates were more difficult to prepare because the Millipore filters clogged rapidly; therefore, three days old cultures (dry weight 0.3 g/l) were filtered through Millipore HA with a 5 mm layer of Celite Hyflo-Super-Cel on it.

*Isolation of Extracellular Products.* For the isolation of all substances except the high molecular weight group, filtrates were adjusted to pH 8 and freeze-dried.

Steam-volatile acids were isolated by extracting the solid residue with methanol. The methanol was evaporated and the residue dissolved in water, acidified with $H_2SO_4$ to pH 3, and distilled. Yellow, water-soluble pigments and lipophilic substances were isolated by extracting the freeze-dry residue with methanol and ethyl ether. The combined methanol/ether extract was evaporated and the residue dissolved in water; after acidifying with $H_2SO_4$ to pH 3 the solution was partitioned with ether The resulting water phase contained the yellow pigments, the ether phase contained the lipophilic substances. Further purification of the yellow pigments was achieved by partitioning the water phase with phenol 80%, after which the phenol phase (containing all of the yellow substances) was washed four times with water to remove all traces of salts. The final phenol solution was freeze-dried. Lipophilic substances were obtained by washing the above mentioned ether phase with water, drying it over anhydrous $Na_2SO_4$, and evaporating it under vacuum. High molecular weight substances were isolated by ultrafiltering the filtrate directly through an Amicon PM-30 membrane which retained all substances with a molecular weight $> 30000$.

For all groups of substances "control cultures", prepared in exactly the same way as the real cultures, but not inoculated with algae, were filtered, extracted, etc. as required for the particular group to be isolated. By comparison of the results of the algal cultures with those of the controls it could be decided whether isolated products really originated from the algae.

*Qualitative and Quantitative Determinations.* As far as possible all isolated groups of substances were weighed and the concentration of each in the original filtrate was computed. Volatile acids in the distillate were titrated with NaOH 0 001 N. The yellow pigments were chromatographed on silica gel G (Merck) with the solvent system methanol/ethanol/acetic acid/water (40:30:2 2) Spots were detected in visible light, under UV 254 nm and after spraying with tetrazotized benzidine The lipophilic fraction was chromatographed on activated silica gel G with the solvent system petroleum ether/diethyl ether/acetic acid (55:35 1). Spots were detected with the method of Dudziński (1967); the chromatogram was observed in visible light and under UV 254 and 366 nm. A determination of proteins (Lowry *et al.*, 1951) with bovine albumine as a standard, and carbohydrates (Hewitt, 1958) with glucose as a standard, was carried out on the high molecular weight fraction.

All determinations were repeated several times. The quantitative values and the descriptions of the thin-layer chromatograms are representative for each particular series of determinations.

## Results

Steam-volatile acids were found in both *Chlorococcum* and *Chlamydomonas* filtrates, in concentrations of $9.0 \times 10^{-6}$ N and $8.3 \times 10^{-6}$ N, respectively.

Sephadex G-10 gel filtration of the yellow, water-soluble pigments as described in Kroes (1972) gave identical results for both species: the pigments were eluted as one peak just after the void volume Their molecular weight thus was at least 1000. *Chlorococcum* produced about 1.5 times as much pigments as *Chlamydomonas* in the same volume of culture medium, judged by the height of the peaks. The fractions collected from the Sephadex column were not suitable for subsequent thin layer chromatography because of the excess of NaOH present. A phenol/water partitioning, however, freed the pigments of all electrolytes. The

product thus obtained was weighed: 1.6 mg/l for *Chlorococcum* and 1.1 mg/l for *Chlamydomonas*. Thin layer chromatography resulted in one prominent yellow spot at $R_f$ 0.7 for both species, and some spots only visible under UV with a slightly different pattern for *Chlamydomonas* and *Chlorococcum*, respectively. As the yellow substances gave one peak on Sephadex and one yellow spot on silica gel, they probably are a homogeneous group or even only one substance. They are phenolic in nature as indicated by a positive reaction with tetrazotized benzidine on the chromatogram.

The lipophilic fraction gave rise to serious problems. Determinations of fatty acids in the *Chlorococcum* filtrate in earlier work (Kroes, 1972) were positive and indicated a concentration of 0.5 mg/l. Thin layer chromatography followed by spraying with a combination of reagents specific for fatty acids (Dudziński, 1967) and co-chromatography with known fatty acids clearly proved their presence. But the fatty acid spot on the chromatogram was also found in the "control cultures" in spite of very careful preparation procedures. The source of the contamination was not discovered. There were, however, other, unidentified, components in the lipophilic fraction which were not found in the controls. For *Chlamydomonas*, five spots with $R_f$-values from 0.0 to 0.3 could be detected under UV after spraying with 2',7'-dichlorofluorescein. On the *Chlorococcum* chromatogram three spots were present, likewise between $R_f$ 0.0 and 0.3. *Chlamydomonas* and *Chlorococcum* had one spot in common: that on the origin. It was yellow in visible light and reacted with $FeCl_3$ and $K_3Fe(CN)_6$ to give a blue-green color, an indication for the presence of phenolic compounds (different from the yellow, water-soluble pigments mentioned above).

High molecular weight substances were found in filtrates of both species. When the retentate from the ultrafiltration was freeze-dried, a white fibrous residue was obtained. From its weight, a concentration of

Table 1. *Concentrations of extracellular substances in filtrates of* Chlamydomonas *cultures (dry weight of cells 0.3 g/l) and* Chlorococcum *cultures (dry weight of cells 0.5 g/l). See text for details*

| | *Chlamydomonas* | *Chlorococcum* |
|---|---|---|
| Steam-volatile acids | $8.3 \cdot 10^{-6}$ N | $9.0 \cdot 10^{-6}$ N |
| Yellow, water-soluble substances | 1.1 mg/l | 1.6 mg/l |
| Lipophilic substances | 0.2 mg/l | 0.5 mg/l |
| High molecular weight substances | 7.5 mg/l | 9.0 mg/l |
| with: | | |
| Proteins | 1.0 mg/l | 4.0 mg/l |
| Polysaccharides | 2.5 mg/l | 4.0 mg/l |

9.0 mg/l in the original filtrate was computed for *Chlorococcum* and 7.5 mg/l for *Chlamydomonas*. The protein concentration in the retentate was determined and gave 4.0 mg albumine equivalent for 1 l of *Chlorococcum* filtrate, 1.0 mg/l for *Chlamydomonas*. The carbohydrate concentrations were 4.0 mg/l and 2.5 mg/l glucose equivalent, respectively.

Table 1 gives a summary of the concentrations of the isolated extracellular products.

## Discussion

Many different organic substances are present in natural waters (e.g. Wagner, 1969), but the origin of most of them is unknown. Algae undoubtedly are one of the several possible sources, not only through the disintegration of cells or whole thalli, but also through the liberation of extracellular compounds. Many types of substances are given off into the culture medium by algae (references: see Kroes, 1972). It is not yet known, however, if the liberation of organic substances follows a general pattern in different species, or if the species differ more or less in the products they give off. Therefore, it is necessary to study the whole array of extracellular compounds of many species. A start in this direction was made by the isolation of several groups of substances from *Chlorococcum* and *Chlamydomonas* filtrates; because these species belong to different orders of the green algae, some idea of the general validity of the results might be obtained.

All groups of substances found in the *Chlorococcum* filtrate were also found in the *Chlamydomonas* filtrate. The concentrations in the latter were consistently lower than in the former, but this was probably due to the lower cell density in the *Chlamydomonas* cultures. Within the groups of lipophilic compounds and yellow pigments there were some specific differences, as revealed by the thin layer chromatograms. The other groups (proteins, polysaccharides, volatile acids) were not analyzed further, but it is possible that here, too, differences in composition between *Chlorococcum* and *Chlamydomonas* existed. Thus, as a first approximation, one may say that *Chlorococcum* and *Chlamydomonas* produce the same groups of extracellular substances and that differences are primarily to be found within the groups. It seems probable that the same holds for other green algae.

In nature, the extracellular products of algae may have different ecological functions. The effect on other species of algae was discussed earlier (Kroes, 1972). Perhaps more important is the utilization of algal substances by aquatic bacteria. Wright and Hobbie (1966) and Allen (1969) showed that aquatic bacteria in nature can grow on very low concentrations of organic substances (glucose, acetate). Therefore, many of the extracellular substances of *Chlorococcum* and *Chlamydomonas*

might serve as nutrients for bacteria in natural waters, especially those from the groups of volatile acids, proteins and polysaccharides.

*Acknowledgements.* I want to thank Miss J. Sterrenberg for skilful technical assistance and Prof. Dr. H. F. Linskens for continued interest and support.

## References

Allen, H. L.: Chemo-organotrophic utilization of dissolved organic compounds by planktic algae and bacteria in a pond. Int. Rev. ges. Hydrobiol. **54**, 1—33 (1969).

Dudziński, A. E.: A spray sequence specific for the detection of free fatty acids. J. Chromatogr. **31**, 560 (1967).

Hewitt, B. R.: Spectrophotometric determination of total carbohydrate. Nature (Lond.) **182**, 246—247 (1958).

Kroes, H. W.: Growth interactions between *Chlamydomonas globosa* Snow and *Chlorococcum ellipsoideum* Deason and Bold under different experimental conditions, with special attention to the role of pH. Limnol. Oceanogr. **16**, 869—879 (1971).

— Growth interactions between *Chlamydomonas globosa* Snow and *Chlorococcum ellipsoideum* Deason and Bold: the role of extracellular products. Limnol. Oceanogr. (in press, 1972).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. J. biol. Chem. **193**, 265—275 (1951).

Wagner, F. S., Jr.: Composition of the dissolved organic compounds in seawater: a review. Contrib. mar. Sci. **14**, 115—153 (1969).

Wright, R. T., Hobbie, J. E.: Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology **47**, 447—464 (1966).

H. W. Kroes
Department of Botany
University of Nijmegen
Toernooiveld
Nijmegen, The Netherlands

# CONCLUSIES

## 1. SAMENVATTING VAN DE RESULTATEN

a) De inleidende proeven met acht wiersoorten (Inleiding, paragraaf 3) lieten zien dat bij combinatie van soorten uit verschillende taxonomische groepen, in dit geval groenwieren en diatomeeën, de mengcultures een hogere maximale dichtheid bereikten dan het gemiddelde van de controle cultures. Bij combinaties tussen soorten uit dezelfde groep (diatomee-diatomee of groenwier-groenwier) werd precies het omgekeerde gevonden. Blijkbaar zijn wiersoorten uit verschillende groepen beter in staat samen de ter beschikking staande voedingsstoffen te benutten dan wiersoorten uit dezelfde groep. Bij deze laatste konden de resultaten zelfs in de zin van een lichte wederzijdse remming worden uitgelegd.

b) Uit de proeven met de filtercultuur opstelling (zie eerste artikel) bleek dat *Chlamydomonas* in ongebufferd medium geremd werd door *Chlorococcum*. Hetzelfde resultaat werd verkregen in proeven met mengcultures, waarbij vooral in de laatste fase van de groei een duidelijke remming optrad. Werden echter mengcultures met gebufferd medium gemaakt (pH ca. 8,2) dan was er geen aantoonbare remming. Uit deze gegevens en uit het feit dat bij *Chlorococcum* in aparte cultuur en bij de mengcultuur de pH sterker opliep dan bij *Chlamydomonas* alleen, werd geconcludeerd dat de pH een belangrijke factor was in de remming van *Chlamydomonas* door *Chlorococcum*.

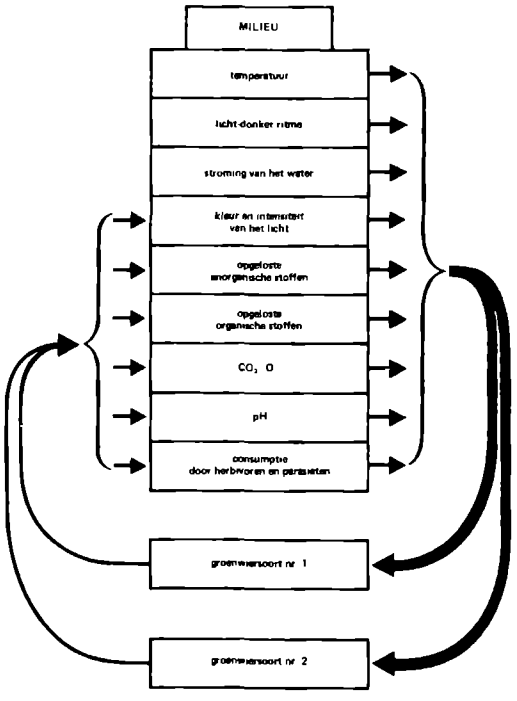
c) Ook extracellulaire stoffen van *Chlorococcum* bleken een – zij het geringe – invloed te kunnen uitoefenen op de groei van *Chlamydomonas* (zie tweede artikel). Vluchtige zuren hadden een duidelijk groeibevorderend effect; lipofiele stoffen remden, vooral in de exponentiële fase, de groei van *Chlamydomonas*; gele, wateroplosbare phenolische verbindingen hadden normaliter geen effect, maar bevorderden de groei sterk in een medium zonder EDTA; de hoogmoleculaire fractie uit het filtraat (polysacchariden en eiwitten) had een bevorderend effect in de eerste dagen van de groei, maar remde tegen het einde van de groeiperiode.

d) Uit de punten b) en c) mag de conclusie worden getrokken dat in een *Chlamydomonas-Chlorococcum* interactiesysteem extracellulaire stoffen een betrekkelijk ondergeschikte rol spelen, temeer omdat ze slechts bij veel hogere concentraties dan die waarin ze in het groeimedium aanwezig zijn effecten vertoonden. De pH van de cultuur is belangrijker in het interactieproces.

e) In *Chlamydomonas*- en *Chlorococcum*-filtraten werden dezelfde stofgroepen gevonden (zie derde artikel). De totale hoeveelheden waren bij *Chlorococcum* steeds wat groter dan bij *Chlamydomonas*. Hierbij moet echter opgemerkt worden dat een rechtstreekse vergelijking niet mogelijk is, omdat uit praktische overwegingen voor *Chlorococcum* oudere culturen werden gefiltreerd dan voor *Chlamydomonas*. Verschillen tussen de twee soorten traden op binnen de groepen van de lipofiele stoffen en de gele, wateroplosbare pigmenten.

## 2. BETEKENIS VAN DE RESULTATEN VOOR DE SITUATIE IN DE NATUUR

Figuur 1 geeft een schematisch overzicht van een water-oecosysteem 'bezien' vanuit twee groenwiersoorten. De nadruk is gelegd op twee tegengestelde aspecten van




Figuur 1

de relaties der wieren met hun omgeving, nl. de invloed die allerlei omgevingsfactoren hebben op de groei van de wieren, en de invloed die de wieren door hun aanwezigheid en groei uitoefenen op de omgeving. Uit het schema is af te lezen dat een eventuele beinvloeding van de éne soort door de andere nooit rechtstreeks verloopt, maar altijd via een beinvloeding van het abiotische gedeelte van het milieu (dit in tegenstelling tot bijv. de relatie predator-prooi). In de meeste gevallen zal een wiersoort meer beinvloed worden door de gesteldheid van de omgeving dan hij zelf de omgeving kan beinvloeden (in het schema aangegeven door de relatieve dikte van de pijlen). Er zijn echter vele uitzonderingen: denk bijvoorbeeld aan de situatie tijdens een wierbloei, aan sterke pH-stijgingen door fotosynthetische activiteit in kleine plassen, enz..

De vraag is nu: kunnen de beinvloeding van het milieu en de daaruit voortvloeiende interacties tussen de soorten zoals die in experimentele situaties gevonden zijn voor *Chlamydomonas* en *Chlorococcum*, ook in de natuur voorkomen? Het spreekt vanzelf dat hierover bij het ontbreken van veldgegevens alleen maar speculaties mogelijk zijn. Eerder is al opgemerkt dat de concentraties van de wieren in de cultures en die van de extracellulaire stoffen bij de interactietests zeer hoog waren in vergelijking met de gemiddelde concentraties in natuurlijk water (zie discussie in eerste en tweede artikel). Het is echter goed denkbaar dat plaatselijk, bijvoorbeeld op de bodem van ondiep water, op grote waterplanten, e.d., de wierconcentraties, en daardoor ook de concentraties van extracellulaire stoffen en de door wieren veroorzaakte verandering van de pH, die van de cultures benaderen. Het feit dat *Chlorococcum*-soorten veel in vochtige grond voorkomen (ARCHIBALD en BOLD, 1970) en dat *C.ellipsoideum* in cultuur sterk de neiging had celaggregaten te vormen en zich vast te zetten op het glas, vormt een duidelijke aanwijzing dat ook in de natuur grotere aggregaten gevormd kunnen worden. Voor *Chlamydomonas* gaat deze veronderstelling waarschijnlijk niet of in mindere mate op. Helaas zijn er geen veldgegevens uit het Ketelven die nadere details over *Chlorococcum* en *Chlamydomonas* opleveren. Recent werk van ALLEN (1971) wijst er echter op dat epiphytische wieren een grote rol kunnen spelen in het oecosysteem van een meer; in het door hem onderzochte meer waren deze wieren verantwoordelijk voor ruim 20% van de totale productie. Bovendien bleek uit dit onderzoek dat er intensieve interacties waren tussen de hogere planten, wieren en bacteriën in deze gemeenschap, vooral wat betreft de uitwisseling van organische stoffen. De experimentele gegevens over *Chlorococcum* en *Chlamydomonas* passen goed in dit beeld van een natuurlijk oecosysteem.

# LITERATUUR

Allen, H L., Primary productivity, chemo-organotrophy, and nutritional interactions of epiphytic algae and bacteria on macrophytes in the littoral of a lake. Ecol.Monogr. **41**, 97-127.

Archibald, P. en Bold, H.C., 1970, Phycological studies. XI. The genus *Chlorococcum* Meneghini Univ. Texas Publ. No 7015.

# STELLINGEN

1

De pH en de ionensamenstelling van het voedingsmedium spelen bij wisselwerkingen tussen wiersoorten een grotere rol dan extracellulaire stoffen van de wieren

Dit proefschrift V W Proctor (1957), Limnol Oceanogr **2**, 125-139, D O Harris (1971), Arch Protistenk **113**, 230-234 G E Fogg (1971), Arch Hydrobiol , Beiheft **5**, 1-25

2

Het 'chlorelline' van Pratt bestaat waarschijnlijk niet

R Pratt et al (1944), Science **99**, 351-352 S Suda (1960), Sci Rep Tōhoku Univ , Ser IV (Biol ) **26**, 189-197 J E Scutt (1964), Amer J Bot **51**, 581-584

3

De filtercultuur methode kan, vooral bij gebruik van 'Spin Filter Flasks', een belangrijk hulpmiddel zijn bij experimenteel oecologisch onderzoek van eencellige organismen

Dit proefschrift P Himmelfarb et al (1969), Science **164**, 555-557 H W Kroes (1973), Oecologia, in press

4

Het begrip 'niche' zoals gedefinieerd door Hutchinson heeft geen eigen wetenschappelijke waarde het is slechts de weergave van een oecologische visie op het soortsbegrip

G E Hutchinson (1965) The ecological theatre and the evolutionary play (Yale Univ Press, New Haven, Conn ), 139 p R H MacArthur (1968), p 159-176 In R C Lewontin (ed ), Population biology and evolution (Syracuse Univ Press, Syracuse, New York)

5

Er zijn niet voldoende argumenten voor de opvatting dat fosfaathoudende wasmiddelen schadelijker zijn voor de toestand van het oppervlaktewater dan niet-fosfaathoudende

W Lange (1967), Nature **215**, 1277-1278 Anonym (1970), Environm Sci Technol **4**, 725-726 J H Ryther & W M Dunstan (1971), Science **171**, 1008-1013 D Mitchell (1971), Science **174**, 827-829

6

Het verschijnsel dat diploïed pollen in staat is een bevruchting tot stand te brengen bij normaliter zelf-incompatibele planten zoals *Petunia hybrida* en *Oenothera organensis* moet niet worden toegeschreven aan concurrentie, maar juist aan samenwerking tussen twee verschillende S allelen.

Straub (1948), Naturwiss. **35**, 23-26. Brewbaker (1954), Genetics **39**, 307-316. Lewis (1960), Proc. Roy. Soc. London, B, **151**, 468-477.

7

Het zou nuttig zijn om, in aansluiting op de indeling van Weisz en Fuller, de benaming *planten* te reserveren voor de mossen, varens en zaadplanten (Metaphyta).

P.B. Weisz & M.S. Fuller (1962), The Science of Botany (McGraw-Hill, New York).

8

Het afwijzen van een biologische/ethologische verklaring van de basis van het menselijk gedrag (met name agressief gedrag) berust niet op wetenschappelijke redeneringen, maar op het vooroordeel dat zo'n verklaring de menselijke waardigheid omlaaghaalt.

K. Lorenz (1963), Das sogenannte Böse (Dr. G. Borotha-Schoeler Verlag, Wien), 391 p. D. Morris (1967), The naked ape (Corgi edition 1971), 219 p. M. Jacobs (1970), Intermediair (Amsterdam) **6**, (19), 1-7. L. Eisenberg (1972), Science **176**, 123-128.

9

Een cryptogamencursus is als een bloemknop die niet opengaat.

10

De waterleiding dient niet als gemakkelijk distributiesysteem voor voedingsbestanddelen en/of geneesmiddelen te worden gebruikt.

11

Autorijden op een openbare weg en vissen anders dan voor voedselvoorziening worden ten onrechte door velen als takken van sport beschouwd.

1 december 1972 H.W. Kroes